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A Method for the Immobilization of Xanthine Oxidase in CA-Alginate Membranes to Measure Hypoxanthine Concentration in Order to Assess Flesh Food Quality

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A METHOD FOR THE IMMOBILIZATION OF XANTHINE OXIDASE IN CA-ALGINATE MEMBRANES TO MEASURE HYPOXANTHINE CONCENTRATION IN ORDER TO ASSESS FLESH FOOD QUALITY

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THESIS ABSTRACT

The consumer's acceptance of food quality is an important factor, especially in Third World Countries which import much of their food. Since flesh food (meat, fish) is shipped to those countries frozen or in cold storage, it is susceptible to temperature fluctuations during shipment, resulting in deterioration of the meat and unacceptable quality. Therefore an acceptable procedure to assess flesh food quality is necessary to protect the consumer and trader and to aid in the manufacture of high quality food products.

Hypoxanthine concentration in flesh food has been a useful index for the assessment of freshness. Therefore, it was selected as the quality control indicator for the development of a rapid and effective method for quality analysis.

An immobilized enzyme analysis system was developed, consisting of a continuous flow reactor, Clark electrode oxidase meter and an immobilized enzyme membrane of 2% Ca-alginate and 1.0 u/ml of xanthine oxidase. The membrane was incubated in 0.05M Tris-HCl buffer, pH 8.4, containing

0.0028% glutaraldehyde. The optimum conditions for the operation of the immobilized enzymes system were pH 7.85, temperature 23°C, circulating buffer flow rate 0.4 ml/min., circulating buffer concentration 0.05M Tris-HCl, substrate (hypoxanthine) prepared in 0.01 M Tris-HCl buffer. The storage stability of the immobilized membrane was more than three months.

The system was evaluated by measuring the hypoxanthine accumulation in meat and fish during storage periods. Meats and fish tested showed that the hypoxanthine concentration increased with increasing refrigerated storage time. Also the system was compared with the colorimetric analysis, and the same pattern of hypoxanthine development was obtained with both analyses.

Comparisons for beef and lamb during cold storage found that sensory evaluation especially as an edibility index, and hypoxanthine concentration, indicated that the majority of potential consumers would purchase and cook those qiven samples of meats, in which the hyponxanthine concentrations were low. When acceptability declined with increased storage time, the hypoxanthine concentration increased.

This analysis procedure was simple, rapid, inexpensive and may prove to be an alternative or adjunct

to the more time consuming and expensive colorimetric analysis, which may also encourage the extension of this work to other meat and fish and their products.

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PREFACE

This dissertation is written to conform with the Graduate School Manuscript Thesis Plan. The text consists of three manuscripts which are written according to the guidelines for publication in the Journal of Food Science.

TABLE OF CONTENTS

Thesis Abstract ii

Acknowledgements v

Preface vi

Table of Contents vii

List of Tables ix

List of Figures x

Manuscript I - A Method for the Immobilization of Xanthine Oxidase on Ca-alginate Membrane to Measure Hypoxanthine Concentration.

I. Introduction 3

II. Materials and Methods 7

III. Results and Discussion 14

IV. Conclusion 23

V. References 60

Manuscript II - Immobilized Xanthine Oxidase Membrane Method for the Measurement of Hypoxanthine

I. Introduction 67

II. Materials and Methods 70

III. Results and Discussion 74

IV. Conclusion 76

V. References 80

Manuscript III - Immobilized Xanthine Oxidase Membrane Method for the Measurement of Hypoxanthine and the Assessment of Meat Quality.

I. Introduction 84

II. Materials and Methods 87

III. Results and Discussion 92

IV. Conclusion 97

V. References 112

Appendix I Literature Review 116

Bibliography 130

LIST OF TABLES

MANUSCRIPT I

1 - Oxidase meter reading and time for a standard sample solution..... 25

2 - Oxidase meter reading and time for a standard sample solution..... 26

3 - Oxidase meter reading and time for a standard sample solution..... 27

4 - Different kinds of algin for preparing membrane..... 28

5 - Comparison of different stabilizing procedures affecting the immobilized enzyme membrane response..... 29

6 - Effect of glutaraldehyde incubation time on the response of immobilized the XOD membrane.....30

7 - Effects of circulating buffer concentration on the XOD enzyme sensor response.....31

MANUSCRIPT II

1 - Comparison of hypoxanthine concentration in fish using immobilized enzyme analysis and colorimetric analysis..... 77

MANUSCRIPT III

1 - Edibility index, odor and organoleptic classes of raw lamb meat..... 98

2 - Edibility index, odor and organoleptic classes of raw beef meat..... 99

3 - Comparison of hypoxanthine concentration using immobilized enzyme analysis and colorimetric analysis for lamb meat..... 100

4 - Comparison using immobilized enzyme analysis and colorimetric analysis for lamb meat..... 101

LIST OF FIGURES

MANUSCRIPT I

1 - The YSI-Clark 2510 electrode and the procedure for fixing the immobilized enzyme membrane and dialysis membrane to the electrode..... 35

2 - Scheme of the electrode and the block diagram system for hypoxanthine determination..... 37

3 - Oxidase reading for a standard sample solution.... 39

4 - Five immobilized membranes with various concentrations of xanthine oxidase..... 41

5 - Effect of various concentrations of glutaraldehyde on immobilized enzyme membrane nanoamp peak height response..... 43

6 - Effect of substrate concentration on the immobilized XOD response..... 45

7 - Effect of injection volume on the response of immobilizede XOD sensor..... 47

8 - Effect of temperature on the reaction rate of an immobilized XOD membrane..... 49

9 - Stability of XOD immobilized membrane at 23°C..... 51

10 - Effect of the reactor flow rate on the XOD enzyme sensor response..... 53

11 - PH profile of the XOD enzyme response to hypoxanthine in Tris-HCL buffers..... 55

12 - Typical standard curve for the hypoxanthine assay using immobilized enzyme analysis..... 57

13 - A comparison between the hypoxanthine concentration determined by the immobilized enzyme analysis and by the colorimetric enzyme analysis..... 59

MANUSCRIPT II

1 - Patterns of hypoxanthine acumulation in Whiting during iced storage as determined by immobilized XOD membrane and colorimetric method..... 79

MANUSCRIPT III

1 - The evaluation sheet used by sensory panelists to determine the odor, color and appearance and acceptability of raw meat.....103

2 - Odor and color and appearance evaluation of raw lamb meat stored at 2-4°C.....105

3 - Odor and color and appearance evaluation of raw beef meat stored at 2-4°C.....107

4 - Patterns of hypoxanthine accumulation in lamb during 2-4°C storage, when analyzed by immobilized XOD membrane sensor and colorimetric method.....109

5 - Patterns of hypoxanthine accumulation in beef during 2-4°C storage, when analyzed by XOD membrane sensor and colorimetric method.....111

MANUSCRIPT I.

A METHOD FOR THE IMMOBILIZATION OF XANTHINE OXIDASE ON CA-ALGINATE MEMBRANE TO MEASURE HYPOXANTHINE CONCENTRATION.

ABSTRACT

Clark electrode, oxidase probe, oxidase meter, and batch reactor with free enzymes, an analysis procedure to measure hypoxanthine concentration was developed. An immobilized xanthine oxidase membrane was developed for measuring hypoxanthine concentration in flesh food. Different types of algin were compared to choose the best agent for membrane manufacture. The effects of $CaCl_2$ concentration on the shape of the membrane and the thinning of the gel membrane were tested. The desired concentration of xanthine oxidase enzyme to immobilize in the membrtane was obtained. The membrane was attached to the oxidase probe, and tried with both a batch reactor and a continuous flow reactor. The influence of glutaraldehyde concentration on the stability of immobilized xanthine oxidase was studied. The effects of assay conditions on the response of the enzyme sensor were studied and evaluated.

A system of continuous flow reactor, Clark electrode, oxidase meter and immobilized enzyme membrane of 2% Ca alginate and 1.0 u/ml of xanthine oxidase, incubated in 0.05 M Tris-HCl buffer, pH 8.4, containing 0.0028% glutaraldehyde gave the best results. The optimum

conditions for the assay were PH 7.85, temperature 23°C, flow rate 0.4 ml/min., and a circulating buffer concentration 0.05 M Tris-HCl. The enzyme sensor could be used for more than 150 assays under these conditions. The storage stability of the immobilized membrane was more than three months at 4°C. Finally, standard sample solutions of hypoxanthine were analyzed and compared, using the immobilized enzyme analysis and colorimetric analysis, over the range of 0-10 ug/ml. The results showed a high degree of correlation between the two methods.

INTRODUCTION

Hypoxanthine, a metabolic by-product of ATP breakdown during autolysis, accumulates in the fish and meat tissues and increases steadily until a maximum is reached. It then decreases as bacterial degradation occurs. If little or no hypoxanthine is present, the fish and meat is considered to be fresh. Thus hypoxanthine can be used as an index of fish and meat quality.

A Colorimetric enzyme assays for the measurement of hypoxanthine concentration in flesh food (meat and fish) have been established and proven to be useful in the assessment of flesh food freshness in quality control (Pizzocaro, 1978, Cattaneo et al., 1979; Platz et al., 1978; Burt et al., 1968; Jahns 1975; Beuchat, 1973; Jacober et al., 1981). This method however is time consuming and expensive.

In recent years, a great deal of interest has been shown in the applications of immobilized enzymes, because they are reusable and open the way to continuous processing (Beeby, 1983). Different immobilization techniques and procedures have been used and discussed (Carr et al., 1980; Hultin, 1974; Klibonov, 1983; Lasking

et al., 1984; Mosback, 1976; Trevan, 1980; Wingard, 1972; Zaborsky, 1973). These immobilization techniques and procedures were classified according to the reactions or processes. 1) covalent attachment of enzymes to solid supports, 2) adsorption of enzymes on solid supports, 3) entrapment of enzymes in polymeric gels, 4) encapsulation of enzymes. Although a number of enzyme immobilization methods have been studied, no one method is ideal, since each method has specific advantages and disadvantages. Therefore, in practice, it is necessary to find a suitable method and conditions for the immobilization of a particular enzyme in light of the intended application (Chibata, 1978).

Several different materials have been used to immobilize enzymes by entrapment method. Among these are cellulose triacetate, collagen, k-carrageenan, algin, agar, and polyacrylamide.

Recently, most attention has been focused on algin, the polysaccharide extracted from brown seaweeds, and the reaction wtih calcium to form Ca-alginate gels, especially for producing ethanol (Hahn-Hagerdal, 1984; Kierstan and Bucke, 1977; Klein and Kressdorf, 1983; McGhee et al., 1984; McGhee et al., 1982; Williams and Munnecke, 1981; White and Portno, 1978), producing citric acid (Vaija et al., 1982; Eikmeier and Rehm, 1984), lactose hydrolysis

(Jacober and Rand, 1984) and the study of banana enzymes (Glass and Rand, 1982).

The reason for the previous researchers choosing alginate immobilization is its simplicity. Futhermore, the immobilization reagents are of low cost and safe, making the procedure attractive for large scale application; also the immobilization procedure is rapid and mild (Kierstan, 1981; Brodelius, 1984; Klibanov, 1983).

Most of the forms of immobilization were in bead form, which cannot be attached to electrodes for research. At this time there have been no reports on alginate membrane form preparation.

A few years ago, the use of biochemical electrodes having bio-specificity attracted considerable attention for the checking or control of the concentration of the metabolites in body fluids. This bio-specificity was based on enzymes, and immobilized enzymes were used in many cases. These electrodes were suitable for the measurement of substrates, coenzymes, and inhibitors of an enzyme, and were called "enzyme electrodes" or "microbial electrodes" when microbial cells were used (Chibata, 1978). Clark and Lyons (1962) proposed the first amperometric enzyme electrode. Since then the literature reviewed shows numerous references to enzyme electrode

production and use (Olson and Richardson, 1974; Hasselberger, 1978; Car and Bowrers, 1980; Wingard et al., 1981; Laskin et al., 1984).

Recently, biological electrodes combined with immobilized membranes have been developed and proven to be a useful method for measuring different compounds which is also quick and simple (Taylor et al., 1977; Updike and Hicks, 1967; Guilbaut and Lubrano, 1972; Guilbaut and Hrabankaoua 1970 a, b; Guilbaut and Shu, 1971; Aizawa et al., 1974; Satoh et al., 1976; Leon et al., 1976; Huntington, 1978; Kayama et al., 1980; Karube et al., 1980, Karube et a., 1 982; Watanabe et al., 1983; Watanabe et al., 1984).

The objective of this study was to develop a procedure and an instrument to measure hypoxanthine concentration, and to develop a new method for membrane manufacture which was simple and safe employing algin to immobilize xanthine oxidase.

MATERIALS and METHODS

Materials

Reagents:

Xanthine oxidase [EC 1.2.3.3, from butter milk, grade 1 (50 units)] xanthine and hypoxanthine were obtained from Sigma Chemical Corp., St. Louis, MO.; anhydrous, purified, calcium chloride was purchased from J. T. Baker Chemical Co., Philipsburg, N.J.; glutaraldehyde (50%) was obtained from Eastman Kodak Co., Rochester, N.Y.; and Kelco gel LV. (specially clarified low calcium alginate) was provided by Kelco Co., Clark, N.J. All other materials used in this experiment were reagent grade and obtained from Fisher Scientific Company, Fairlawn, N.J. Deionized distilled water was used throughout.

Equipment:

The YSI Model 25 oxidase meter and YSI-Clark 2510 electrode were obtained from Yellow Springs instrument Co., Inc., Yellow Springs, Ohio. The continuous flow reactor was designed in the Engineering Instrument Shop of the University of Rhode Island, Kingston, Rhode Island; the peristaltic pump was a polystaltic model obtained from Buchler Instruments, Inc., Fort Lee, N.J. The reporting integrator 3390A was obtained from Hewlett Packard,

Avondale, PA. The small flat dish (diameter 4.5 cm and depth 0.5 cm) required for membrane preparation was purchased from Goldman Sciences (No. 4,160,700). The septum injector was purchased from Rainin Instruments Co, Woburn, MA.

Assay of hypoxanthine

Batch Reactor:

The assay was carried out in two attempts. In the first attempt the Clark electrode was stabilized in 20 ml 0.05 M potassium phosphate buffer, pH 7.6. Then the probe was immersed into an equal volume of buffer containing certain concentrations of hypoxanthine, with constant mixing on a magnetic stirrer to make sure that the YSI oxidase meter reading was zero. The zero adjustment was necessary to offset any current generated by interfering substances. A 0.1 ml aliquot of stock xanthine oxidase concentration (50 units) was added at time zero, and readings of nanoamps were taken at appropriate intervals until stabilization occurred. The highest stabilized reading was taken as representing the concentration of hypoxanthine.

In the second attempt a small piece of the immobilized enzyme membrane (about 0.7 cm diameter) was cut by a Pasteur Pipet and attached to the Clark

Electrode, as shown in Figure 1. The assay procedure was performed as described above, except there was no need to add 0.1 ml of xanthine oxidase; and the buffer was changed to 0.05 M Tris-HCl, pH 8.4 because the presence of phosphate tended to disrupt membrane structure (Kierstan and Bucke, 1977).

Continuous flow reactor:

The continuous flow reactor (reaction chamber), designed in the Engineering Department workshop at the University of Rhode Island, Kingston, Rhode Island, was similar to the one described by Watanabe et al. (1983), with some modification as shown in Figure 2. In the initial trials, the buffer solution was 0.05 M Tris-HCl, pH 8.4 buffer; later the pH of the buffer was changed to 7.85. The buffer solution was transfered continuously to the reaction chamber by a peristaltic pump through 0.3125 cm outer diameter teflon tubing. Sufficient time was allowed for the probe to stabilize, which indicated when a steady state was reached. Initially, readings from the oxidase meter (nanoamp) or the recorder were difficult to interpret especially for values close to each other. It was found that connecting the integrator to the oxidase meter, instead of the recorder, was more accurate and

sensitive, even to small changes in the responses using peak height operation. Because it was found that peak height was increased by increasing the substrate concentration, peak height was used as a measure of enzyme sensor response. In addition the integrator could function as an analytical computer for reporting the results.

After the oxidase meter reading became steady, 100 ul of the hypoxanthine solution was injected into the flow line just ahead of the enzyme reactor. The maximum current generated by the xanthine oxidase probe response was directly proportional to the hypoxanthine concentration.

Ca-alginate Membrane Preparation:

Different kinds of algin and different $CaCl_2$ concentrations were tested to develop a new method for membrane manufacture. The preparation of algin solution by stirring and by blending were compared. The effects of adding and spraying $CaCl_2$ on the shape of the membrane and on its thinning were studied. A flat glass plate and a flat dish with edges to control the desired volume and shape of the gel membrane were evaluated.

Immobilized Xanthine Oxidase:

The desired concentration of xanthine oxidase for preparing the membrane and the effect of glutaraldehyde concentrations as a cross linkage on the stability of immobilized enzyme membrane were studied.

Membrane Assay Conditions:

The effect of substrate (hypoxanthine) concentration, pH, flow rate, temperature, injection volume, and buffer concentration on the enzyme sensor response were studied and evaluated.

Xanthine oxidase assay:

The activity of stock xanthine oxidase concentrate, in units/ml, was determined each time a new membrane was prepared, utilizing a procedure described by Sigma Co.

To 1.9 ml buffer, 0.15M potassium phosphate, pH 7.6, and 0.1 ml of 0.2--0.3 unit/ml xanthine oxidase, 1.0 ml of 0.002% xanthine was added. (Both xanthine oxidase and xanthine were prepared in 0.15 M potassium phosphate buffer, pH 7.6). After incubating for 2.5 minutes at 25^{o}C, the increase in the mixture absorbance at 290 nm was measured in a Bausch and Lomb Spectronic 21 spectrophotometer. The blank consisted of 2.9 ml buffer and 0.1 ml enzyme. To determine the enzyme activity

equation (1) was used.

Equation 1. Determination of xanthine oxidase activity.

$$\text{Enzyme units/ml} = \frac{\text{A/min} \times 1000 \times \text{ml reaction mixture} \times \text{dilution of enzyme}}{\text{molar absorbancy of uric acid} \times \text{ml enzyme in reaction mixture}}$$

where: enzyme units/ml = enzyme activity per ml of the stock concentrate solution

A/min. = change in absorbance per ml reaction mixture per minute

ml reaction mixture = 3.0 ml

molar absorbance of uric acid = 1.22×10^4 cm^{-1}

ml of enzyme in reaction mixture = 0.1

Hypoxanthine Assay Through Colorimetry:

To construct the hypoxanthine standard (calibration) curve, a method described by Jahns and Rand (1977) and modified by Jacober et al. (1981) was utilized. Standard sample solutions of hypoxanthine consisting of 0 to 10 ug/ml in 0.15M potassium phosphate, pH 7.6, were prepared. To 0.5 ml of each solution was added 2 ml of a 2,6-dichloroindophenal-xanthine oxidase solution (DIP/XOD). This solution consisted of 3 parts 23 mg/ml DIP and 1 part 0.02-0.03 unit/ml XOD. (Both were prepared in a 0.15M potassium phosphate buffer at pH 7.6). After incubating

for 2.5 minutes at 25°C, the decrease in the mixture's absorbance at 600 n.m. was measured in a Bausch and Lomb Spectronic 21 spectrophotometer. The blank consisted of 2.5 ml of buffer, whereas the reference solution was 2.0 ml DIP/XOD and 0.5 ml of phosphate buffer.

Results and Discussion

In preliminary trials many steps were tried in order to develop this system. In the first step, a YSI Clark 2510 electrode and a YSI 25 oxidase meter, were tested to see if they were able to measure hypoxanthine. A standard sample solution, consisting of 0-10 ug/ml of hypoxanthine and 0.2-0.3 u/ml xanthine oxidase, was prepared and tried in the batch reactor. The results (as shown in Table 1 and Figure 3) indicated that the resulting current was directly proportional to hypoxanthine presence. The reaction time seemed long, especially with the higher amounts of hypoxanthine, but because in the bulk solution the enzyme has relatively less concentrated, a longer time was required to attain a steady state. Therefore, the enzyme concentration was increased to 0.64 unit/ml and 0.90 u/ml as shown in Table 2 and Table 3 respectively. The results showed that increasing the enzyme concentration shortened the time required to attain a steady state.

At the conclusion of this step, the approach showed promise using the YSI Clark 2510 electrode and YSI Model 25 oxidase meter as a tool for measuring the hypoxanthine concentration.

The second step was the immobilization of the xanthine oxidase. A simple, rapid, low cost and applicable method for immobilization was preferable. Alginate gels (using the entrapment method) seemed to meet these criteria (Klibanov, 1983; Kierstan, 1981; Bradelius, 1984).

In order to prepare the membrane much time was spent to obtain a good membrane (thin gel), since the main reaction to form the membrane was between algin and $CaCl_2$. Several articles were reviewed to choose the best concentration of algin and $CaCl_2$ and the effect of their concentration on the diffusion of substrate Ca-alginate gels (Tanaka, 1984; Kierstan, 1981; Kierstan and Bucke, 1977; Cheetham et al., 1979). They found that substrates with a molecular weight (MW) less than 2×10^4 could diffuse freely into and from Ca alginate gel beads. Also, neither the Ca-alginate concentration in the beads, nor the $CaCl_2$ concentration used in the gel preparation, had any effect on the diffusion of the substrates. The efficiency of retention of large molecular-weight compounds increased when 2% (W/V) sodium alginate was used (Kierstan and Bucke, 1977). Tanaka (1984) found that substrates with small molecular weight (such as glucose) could diffuse into 2% Ca-alginate gel beads as freely as water. Therefore, a 2% algin concentration was chosen.

Since there are different kinds of algin, the following products of the Kelco Company -- Kelmar, Kelco Gel HV, Kelco Gel LV, Kelcosal, Keltone, Kelgin RL, Kengin LV and Kelset -- were examined to choose the one most suitable for the membrane. Each algin was dissolved in distilled water by stirring until all the powder dissolved. Then, each solution was spread on a flat glass plate, and $CaCl_2$ was added until the gel formed. It was found that Kelmar needed small amounts of $CaCl_2$ to gel but did not make a thin gel (membrane); it could be good to use in preparing a bead. Kelcosol did not form a good gel with $CaCl_2$. Kelset was self gelling and became softer with the addition of $CaCl_2$. Kelco Gel HV, Kelco Gel LV, Kelgin RL, Kelgin LV and Keltone formed gels with a moderate amount of $CaCl_2$. It was decided that Kelco gel LV provided the best uniformity (shape), so it was chosen for development of the membrane (Table 4). It was found that preparing the algin solution by adding the algin to distilled water in a Waring Blender Jar at high speed until a uniform misture was obtained was quicker than dissolving by stirring. The blended mixture was stored at $4^{o}C$ until needed. It was difficult to get a soft and thin gel (membrane) by just adding $CaCl_2$ to the alginate solution. So a new attempt was tried: spraying the $CaCl_2$ by an atomizer connected to an air pressure pump. Also, a small

flat dish with edges was found to be important to control the desired volume and shape of the gel membrane, and a Gelman Sciences small flat dish with dimensions of 4.5 cm. diameter and 0.5 cm. depth was found to be ideal These changes dramatically improved the membrane preparation procedures, and a thin gel could be formed, which was used throughout the study.

The effect of the $CaCl_2$ concentration on the shape of the membrane and the thinning of the gel membrane was tested. Concentrations of 1, 2, 3, 4, 5, and 6% $CaCl_2$ were prepared. It was found that at low concentrations (1-4%), after spraying and adding extra $CaCl_2$, the gel membranes had shrunk to almost half their size and increased in thickness. However, at 5% and 6% $CaCl_2$ level, there was no decrease in size and a uniform gel membrane formed. Therefore, 5% $CaCl_2$ was used for preparing the membrane. Also, it was found that spraying with $CaCl_2$ several times over a ten-minute period, and then covering the membrane with $CaCl_2$ solution for 20 minutes more gave the best results for membrane formation.

Determination of the effectiveness of the membrane and selection of the desired concentration of xanthine oxidase for preparing the membrane was studied. Different gels containing xanthine oxidase concentrations of (0.2, 0.4, 0.6, 1.0 and 2.0 unit/ml, respectively, were prepared

by mixing 3 ml of 2% alginate solution with 9.2-92 ul of stock xonthine oxidase concentrate (21.74 u/ml). These combinations were mixed in the dish by hand and sprayed with 5% $CaCl_2$ solution for 10 minutes. Extra $CaCl_2$ solution was then added to cover the membrane for 20 minutes. The membranes were then washed with distilled water and stored in 0.05 M Tris-HCl buffer pH 8.4, at 4^oC until needed. A small circle of the prepared enzyme membrane was cut with a Pasteur Pipet, attached to the probe, and covered with a dialysis membrane (Figure 1); and the analysis was conducted as described for the batch reactor. Similar results as seen in Figure 4 were obtained by using this procedure, as the enzyme concentration increased rom 0.2-1.0 u/ml the sensor response increased. Any further increasing of enzyme concentration of 1.0 to 2.0 u/ml produced no further increases in sensor response.

Since the new procedure was working, a reaction chamber (continuous flow reactor) was designed in the Engineering Instrument Shop of the University of Rhode Island as described in the methodology section. A circle of each of the same enzyme membranes prepared earlier, containing (0.2, 0.4, 0.5, 1.0 and 2.0 u/ml of XOD, were cut and attached to the electrode as described above. The immobilized enzyme sensor was attached to the reaction

chamber as shown in Figure 2. The results, as seen in Figure 4, indicated that the new system was working and was a usable procedure. The desirable enzyme concentration was 1.0 u/ml, since higher levels of XOD produced no further increases in peak height response.

The only weakness of this immobilization technique has been reported that the enzyme often leaked from the support. It was felt that this could be overcome by treatment of the entrapped enzyme (membrane) with a cross linking reagent. Glutaraldehyde has been the most recommended and usable compound of this type for immobilization procedures due to the low cost and minimal effects on enzymes (Klibonov, 1983; Watanable et al., 1983; Watonable et al., 1984; Messing, 1975; Zaborsky, 1973; Jacober and Rand, 1980; Chibata, 1978).

Therefore, an initial trial was made to see which was the best way to prepare XOD immobilized membrane stabilized with glutaraldehyde. Glutaraldehyde (0.0028%) was mixed with the alginate solution and the enzyme before gelation or by incubating the membrane in the gluraldehyde solution for 24 hours after gelation. A control membrane was prepared (no glutaraldehyde). The results, Table 5, show that the enzyme sensor response of the immobilized XOD in the alginate membrane were improved with the use of glutaraldehyde. Incubating the XOD membrane in 0.05 M

Tris-HCl buffer solution, pH 8.4, containing glutaraldehyde virtually doubled the activity, whereas just mixing it with glutaraldehyde prior to formation only slightly improved the response and made the membrane (gel) tough and brittle. Conducting immobilization of the XOD on ice, instead of at room temperature, did not improve the response. Finally, 2 ml of 2% alginate solution was mixed with 46 ml of stock XOD concentrate (21.74 u/ml) in the procedure, instead of 3 ml alginate, and gave a better membrane. Therefore, a volume of 2 ml of 2% alginate was used in all subsequent experiments.

Due to the success with glutaraldehyde, the optimum concentration was studied with immobilized enzyme membranes which were incubated for 24 hours in 0.05 M Tris-HCl buffer solution, pH 8.4, and contained the following concentrations of glutaraldehyde: 0.002, 0.0028, 0.004 and 0.008% . Figure 5 shows the results obtained by utilization of the immobilized enzyme membranes treated with various glutaraldehyde concentrations. The enzyme sensor response on the same substrate concentration increased with increasing glutaraldehyde from 0.002% to an optimum at 0.0028%. At higher levels the response decreased, which may be due to an increase of crosslinked networks which reduced the substrate diffusion into the membrane or the result of enzyme inactivation with

additional polymerization reactions. The optimum glutaraldehyde value of 0.0028% was chosen as the cross linkage reagent concentration for stabilization of the immobilized enzyme membrane in all other studies.

The effect of glutaraldehyde incubation time on the XOD immobilized membrane was also studied. Two membranes were prepared and incubated for 12 and 24 hours. As seen in Table 6, incubation of a XOD membrane in 0.05 M Tris-HCl buffer, pH 8.4, for 12 hours gave the best enzyme sensor response and reduced the preparation time.

The effects of substrate concentration and injection volume on the response of the enzyme sensor were studied. Hypoxanthine solutions of 0.001, 0.002, and 0.003% were prepared and 25, 50, 100 ul of the hypoxanthine solution were injected. The results, as shown in Figure 6 and Figure 7, indicate that the response of the enzyme sensor increased with increasing both the substrate concentration and injection volume.

Figures 8 and 9 illustrate the effect of temperature on the reaction rate and the stability of enzyme activity. A temperature of 23^{o}C was chosen as the reaction temperature, because the enzyme sensor response was the highest value and the enzyme activity (stability) decreased very little, even after 16 hours of constant use.

As one of the factors to be considered in the design of an enzyme reactor, the relation between the flow rate and the response of the enzyme sensor had to be studied. Figure 10 shows this relationship. Above 0.4 ml min^{-1}, the XOD sensor response decreased due to insufficient time for the reaction.

The effect of pH on the XOD sensor reaction rate was evaluated between pH 7.6-8.5, as seen in Figure 11. The maximum response was at pH 7.85.

The effect of circulating buffer concentration on the response of the XOD enzyme sensor was studied as shown in Table 7. The results indicated a substantial enhancement using 0.05 M for the circulating buffer and using 0.01 M for substrate preparation.

Standard sample solutions of hypoxanthine were analyzed and compared, using the immobilized enzyme analysis procedure developed here and the standard colorimetric enzyme analysis. As seen in Figure 12 a linear relationship was obtained between hypoxanthine concentration and the peak height responses over the range of 0-10 ug/ml. Comparing this result to the colorimetric analyses in Figure 13 shows a high degree of correlation over the same range.

Conclusion

The emphasis in this research has been on developing a procedure to measure hypoxanthine concentration and on employing an oxidase meter and Clark electrode oxidase probe as an instrument, when combined with a new method for membrane manufacture which is simple and safe, employing algin.

The oxidase probe and oxidase meter combined with immobilized xanthine oxidase on a Ca-alginate membrane is a simple, easy, as well as relatively quick and inexpensive procedure. The system seems to be a good procedure to study substances that produce or influence production of H_2O_2. The optimum conditions which were established in this study are as follows: pH 7.85, temperature 23°C, sample injection volume 100 ul, flow rate 0.4 ml/min., circulating buffer concentration 0.05 M Tris-HCl, and substrate buffer 0.01 M Tris-HCl buffer. The storage stability of the immobilized membrane was found almost constant every time, being used for more than 3 months at 4°C.

The Ca-alginate membrane prepared in this study could be used to immobilize any enzyme or multi-enzyme. The

immobilized enzyme membrane may be coupled with the appropriate electrode to measure a chosen compound, and readily employed for routine analysis.

Table 1. Oxidase meter reading and time for a standard sample solution consisting of 0-10 ug/ml of hypoxanthine and 0.3 u/ml of xanthine oxidase (both in 0.05 M potassium phosphate buffer, pH 7.6), at room temperature.

Hypoxanthine ug/ml	Probe Response (nanoamp)	Time (min.)
2.5	4.50	6
5.0	8	6
7.5	12	8
10	15	24

Table 2. The oxidase meter readings and times for a standard sample solution consisting of 0-10 ug/ml of hypoxanthine and 0.64 u/ml xanthine oxidase (both in 0.05 M potassium phosphate buffer, pH 7.6), at room temperature.

Hypoxanthine ug/ml	Probe Response (nanoamp)	Time (min.)
2.5	5	6
5.0	9.5	6
7.5	13.50	7
10	16.0	7

Table 3. Oxidase meter reading and time for a standard sample solution consist of 0-10 ug/ml of hypoxanthine and 0.9 u/ml xanthine oxidase (both in 0.05 M potassium phosphate buffer, PH 7.6), at room temperature.

Hypoxanthin ug/ml	Probe Response (nanoamp)	Time (min.)
2.5	4	3
5.0	7	4
7.5	13.50	5
10	16.0	5

Table 4. Different kinds of algin for preparing a membrane.

Product	$CaCl_2$ Amound Needed	Gel Hardness Score *
Kelmar	Small	1
Kelcosol	Large	4
Kelset	No need (self gelling)	5
Kelco Gel HV	Moderate	2
Kelco Gel LV	Moderate	3
Kelgin RL	Moderate	2
Kelgin LV	Moderate	2
Keltone	Moderate	2

* Hard (1) to Soft (5)

Table 5. Comparison of different stabilizing procedures affecting the immobilized enzyme membrane response (pH 7.85, 0.002% hypoxanthine), at room temperature.

Membrane	Enzyme Sensor Response (peak height)
Control (H_2O)[a]	150×10^3
Control (H_2O)[b]	145×10^3
Mixed with glutaraldehyde[c]	157×10^3
Incubated in glutaraldehyde[c]	343×10^3

a = prepared at room temperature

b = prepared on ice

c = 0.0028% glutaraldehyde

Table 6. Effect of glutaraldehyde incubation time on the response of immobilized the XOD membrane.

Incubation Time	Enzyme Sensor Response (peak height x 10^3)
12	280
24	220

Table 7. Effects of circulating buffer concentration on the XOD enzyme sensor response (100 ul of 0.002% hypoxanthine, flow rate 0.4 ml/min., pH 7.85, Temp. 23°C.

Circulating buffer concentration	Sample buffer concentration 0.01M	0.05M
0.01	0	0
0.05	167%	15%
0.1	199%	78%

FIGURES

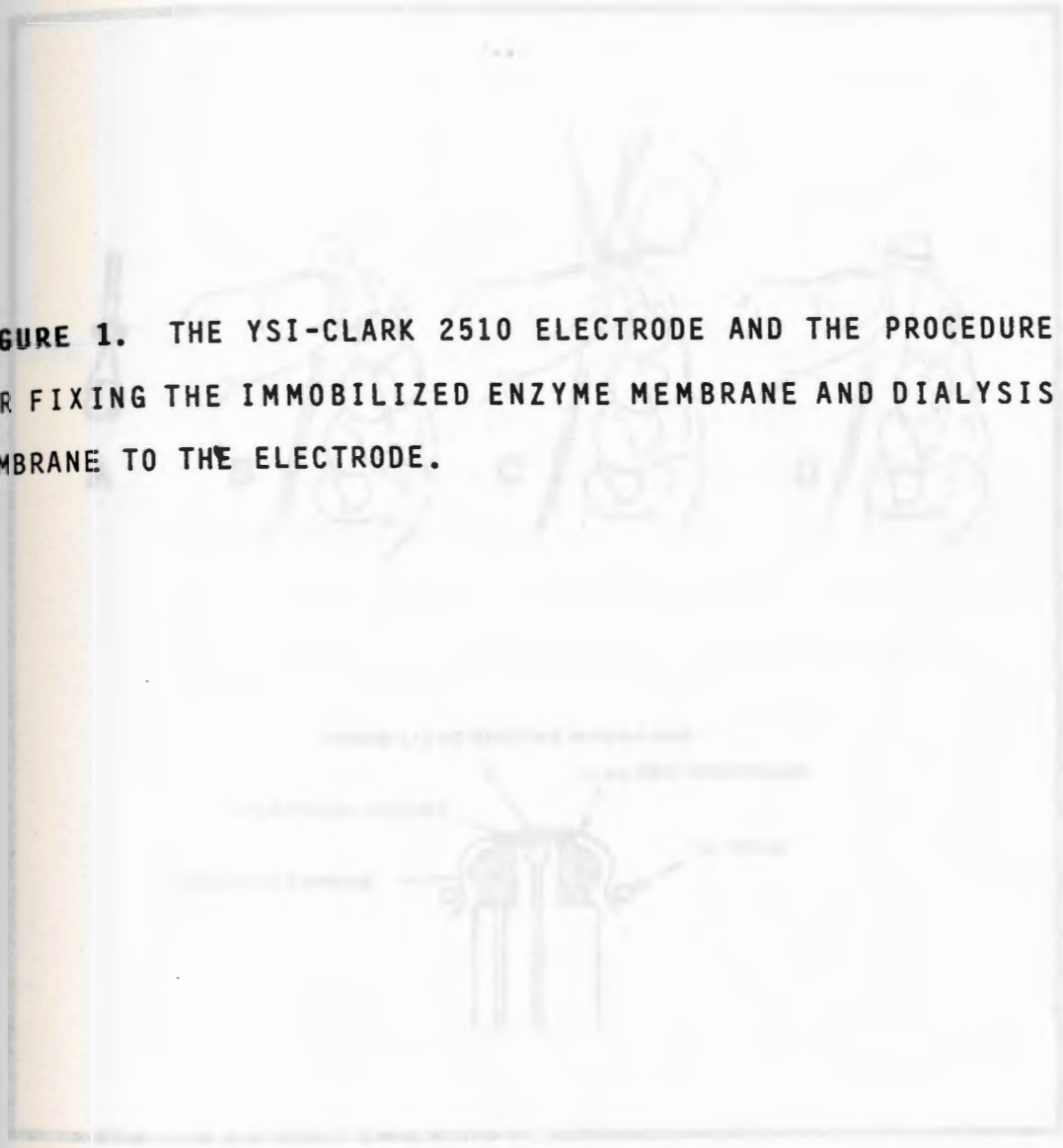
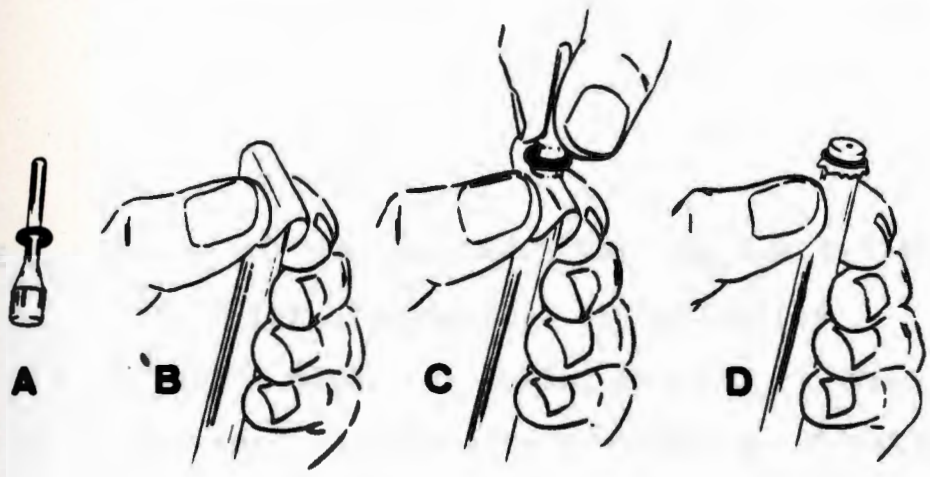


FIGURE 1. THE YSI-CLARK 2510 ELECTRODE AND THE PROCEDURE FOR FIXING THE IMMOBILIZED ENZYME MEMBRANE AND DIALYSIS MEMBRANE TO THE ELECTRODE.


A
B
C
D

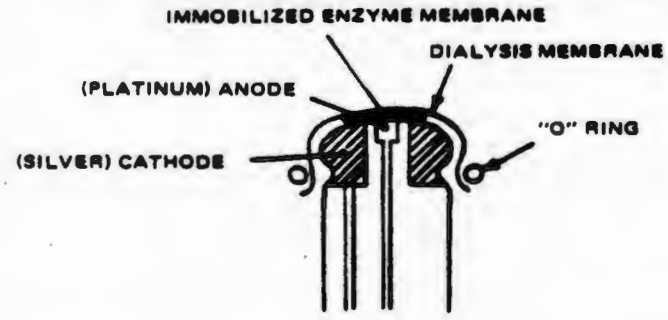
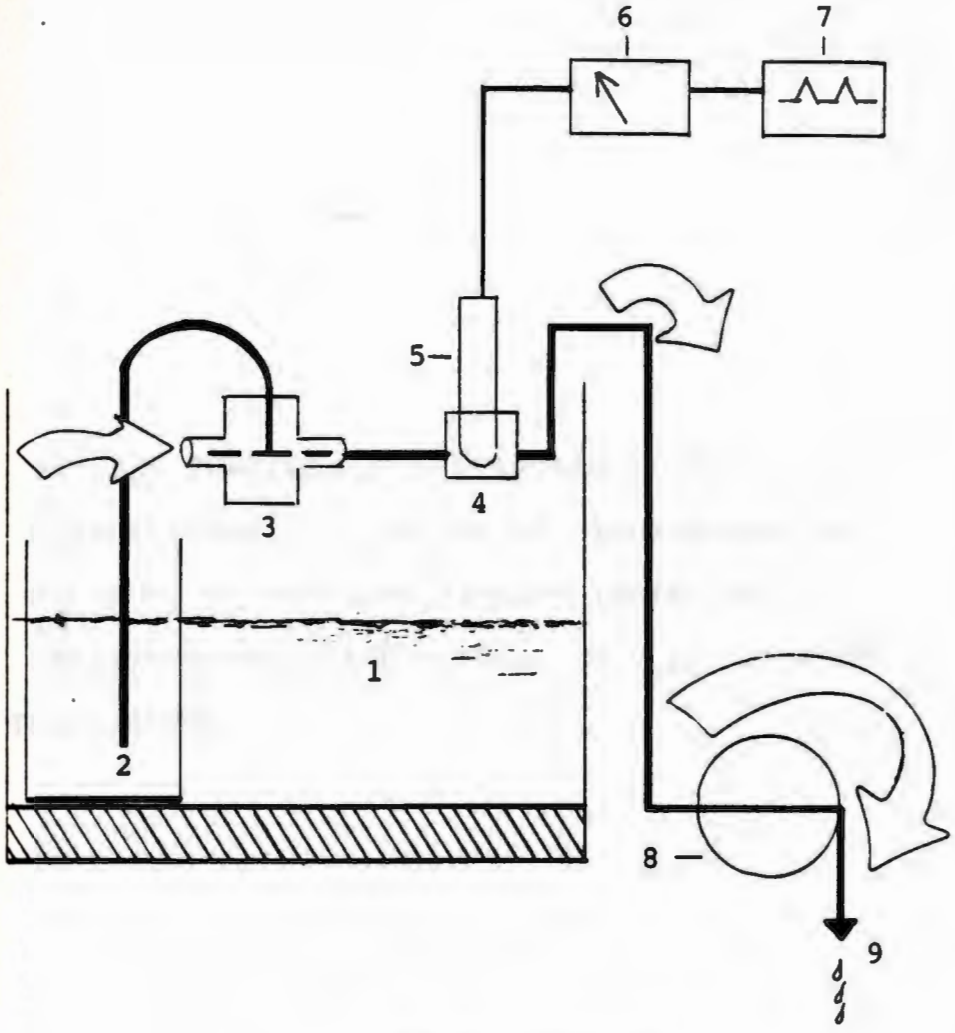
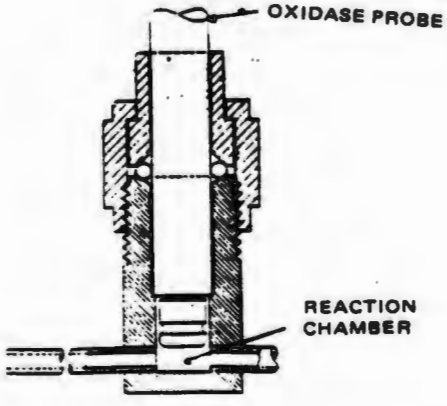

IMMOBILIZED ENZYME MEMBRANE
DIALYSIS MEMBRANE
(PLATINUM) ANODE
"O" RING
(SILVER) CATHODE

FIGURE 2. SCHEME OF THE ELECTRODE AND THE BLOCK DIAGRAM SYSTEM FOR HYPOXANTHINE DETERMINATION.
1. Water Bath, 2. Buffer Beaker, 3. Septum injector, 4. Reaction Chamber, 5. Electrode, 6. Oxidase Meter, 7. Integrator (or recorder), 8. Pump, 9. Waste, 4a. Cross-Sectional View of Oxidase Probe and Reaction Chamber.





4a

FIGURE 3. OXIDASE READING FOR A STANDARD SAMPLE SOLUTION CONSISTING OF 0-10 mG/ML OF HYPOXANTHINE AND 0.3 U/ML OF XANTHINE OXIDASE (BOTH IN 0.05 POTASSIUM PHOSPHATE BUFFER, pH 7.6), AT ROOM TEMPERATURE.

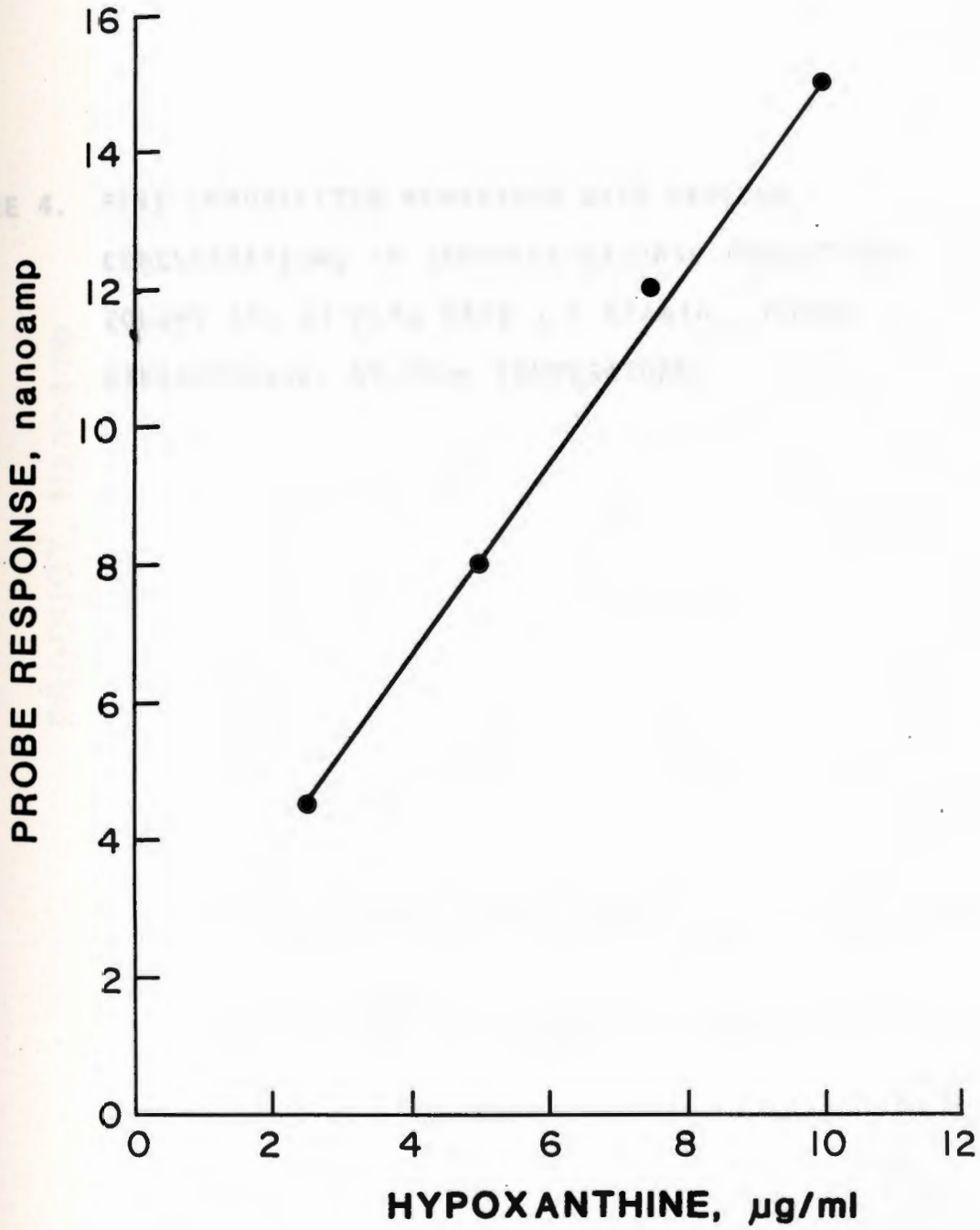
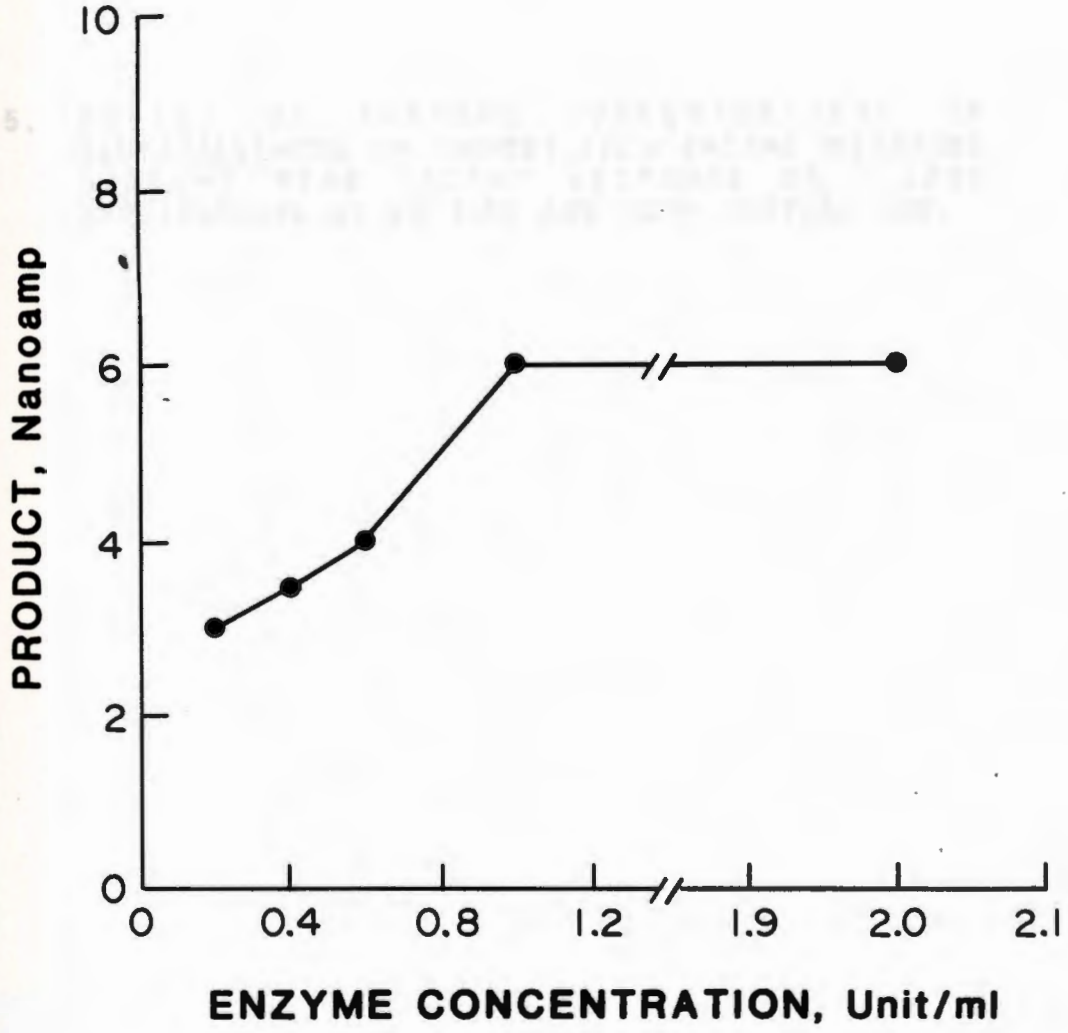

PROBE RESPONSE, nanoamp
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
HYPOXANTHINE, µg/ml

FIGURE 4. FIVE IMMOBILIZED MEMBRANES WITH VARIOUS CONCENTRATIONS OF XANTHINE OXIDASE (INJECTION VOLUME 100 ul FLOW RATE 1.4 ml/min., 0.002% HYPOXANTHINE, AT ROOM TEMPERATURE.


10
8
6
4
2
0
PRODUCT, Nanoamp
0
0.4
0.8
1.2
1.9
2.0
2.1
ENZYME CONCENTRATION, Unit/ml

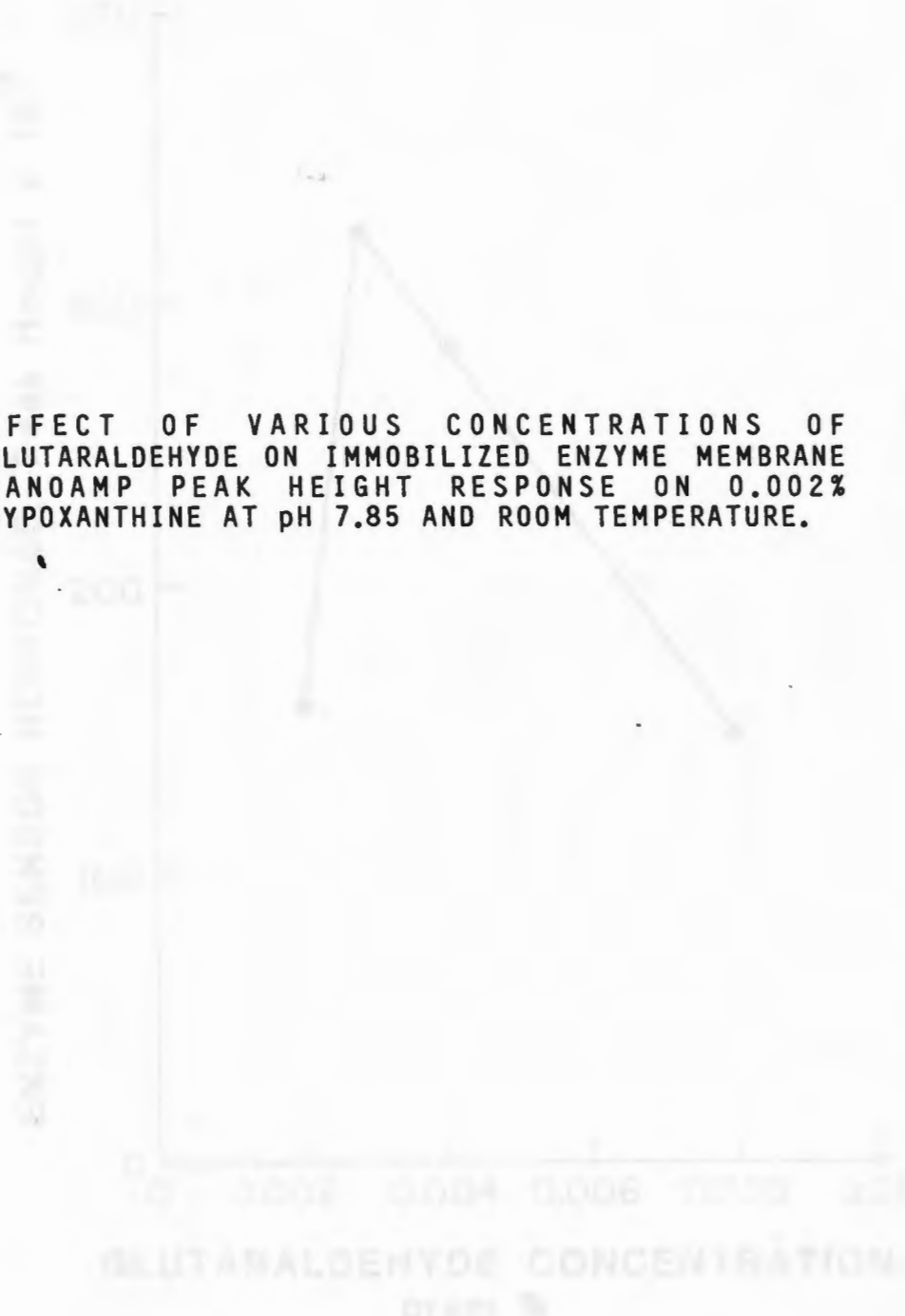


FIGURE 5. EFFECT OF VARIOUS CONCENTRATIONS OF GLUTARALDEHYDE ON IMMOBILIZED ENZYME MEMBRANE NANOAMP PEAK HEIGHT RESPONSE ON 0.002% HYPOXANTHINE AT pH 7.85 AND ROOM TEMPERATURE.

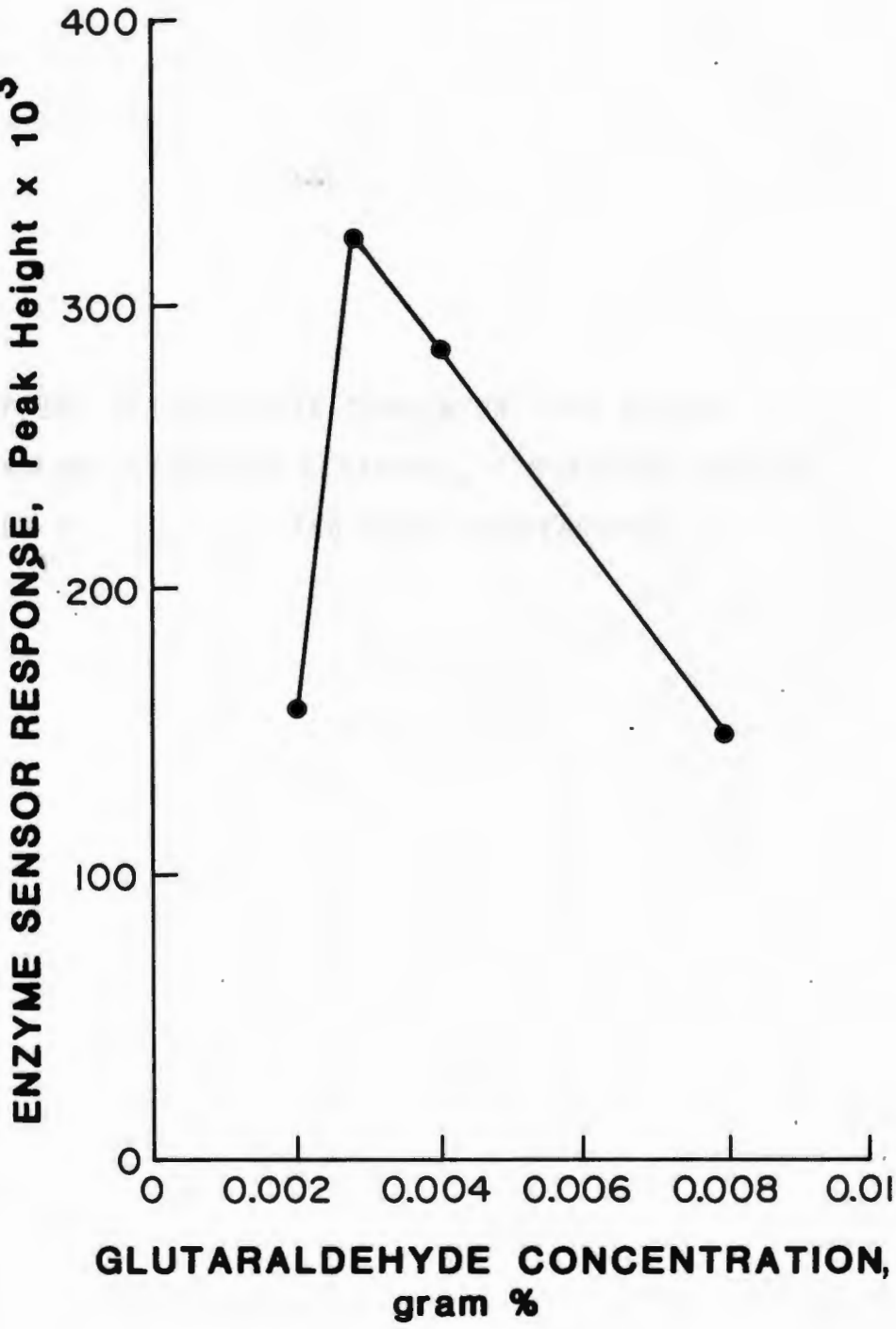

400
300
200
100
0
ENZYME SENSOR RESPONSE, Peak Height x 10^3
0
0.002
0.004
0.006
0.008
0.01
GLUTARALDEHYDE CONCENTRATION, gram %

FIGURE 6. EFFECT OF SUBSTRATE CONCENTRATIONS ON THE IMMOBILIZED XOD RESPONSE, (INJECTION VOLUME, 100 ML; pH, 8.4; AND ROOM TEMPERATURE).


ENZYME SENSOR RESPONSE,
Peak Height x 10^3
300
240
180
120
60
0
0
40
80
120
160
200
TIME, Minutes
○ 0.003% HYPOXANTHINE
▲ 0.002% HYPOXANTHINE
● 0.001% HYPOXANTHINE

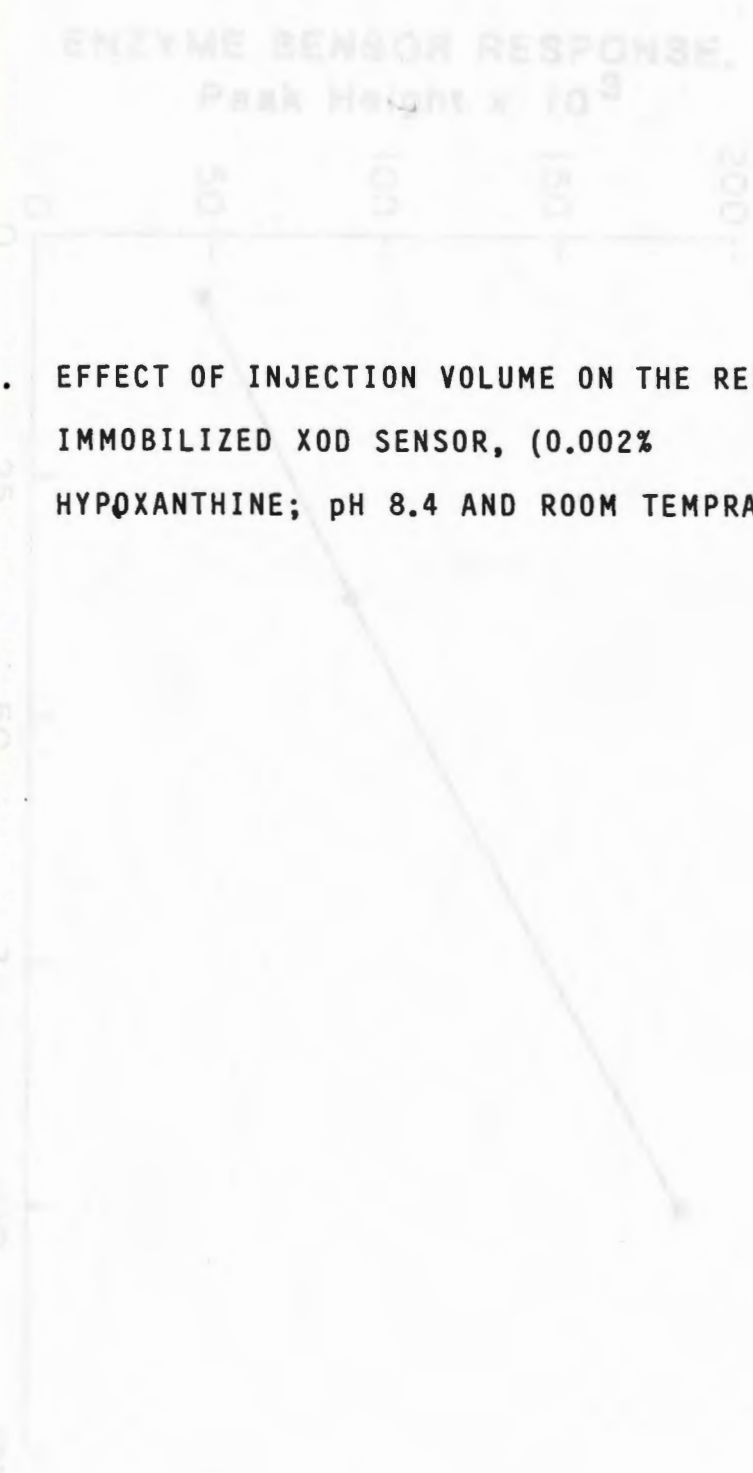
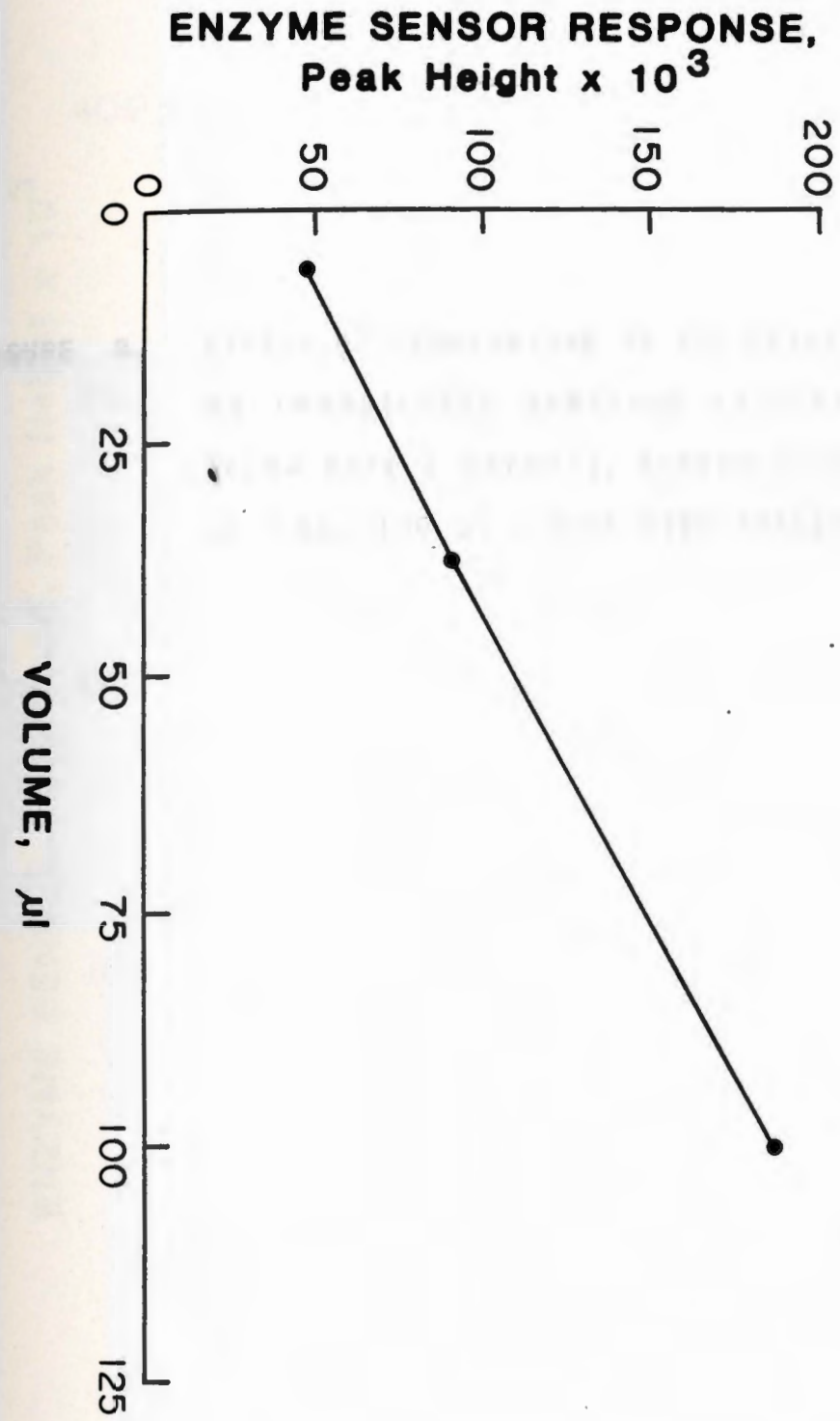


FIGURE 7. EFFECT OF INJECTION VOLUME ON THE RESPONSE OF IMMOBILIZED XOD SENSOR, (0.002% HYPOXANTHINE; pH 8.4 AND ROOM TEMPRATURE).


ENZYME SENSOR RESPONSE,
Peak Height x 10^3
0
50
100
150
200
VOLUME, µl
0
25
50
75
100
125

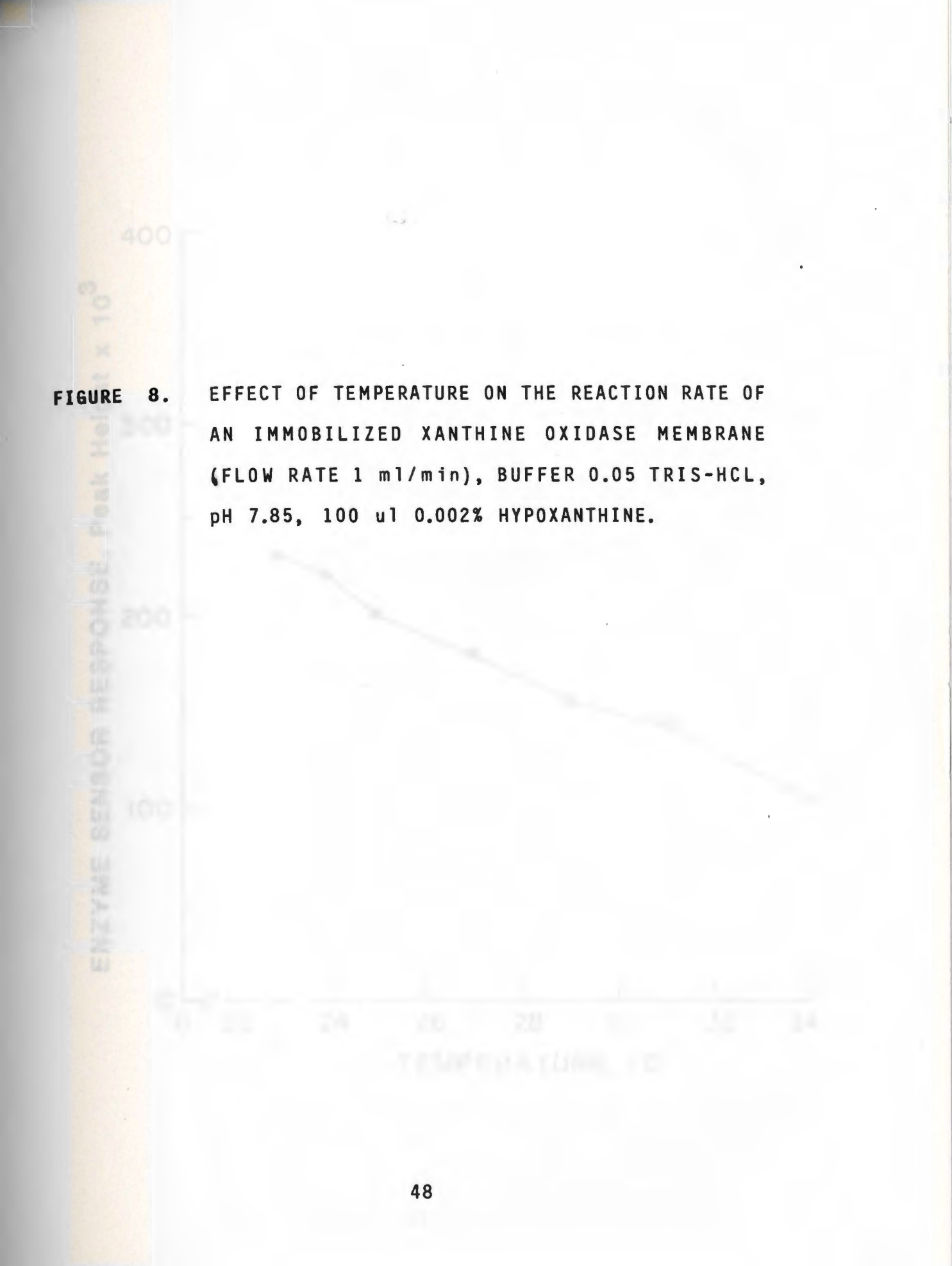


FIGURE 8. EFFECT OF TEMPERATURE ON THE REACTION RATE OF AN IMMOBILIZED XANTHINE OXIDASE MEMBRANE (FLOW RATE 1 ml/min), BUFFER 0.05 TRIS-HCL, pH 7.85, 100 ul 0.002% HYPOXANTHINE.

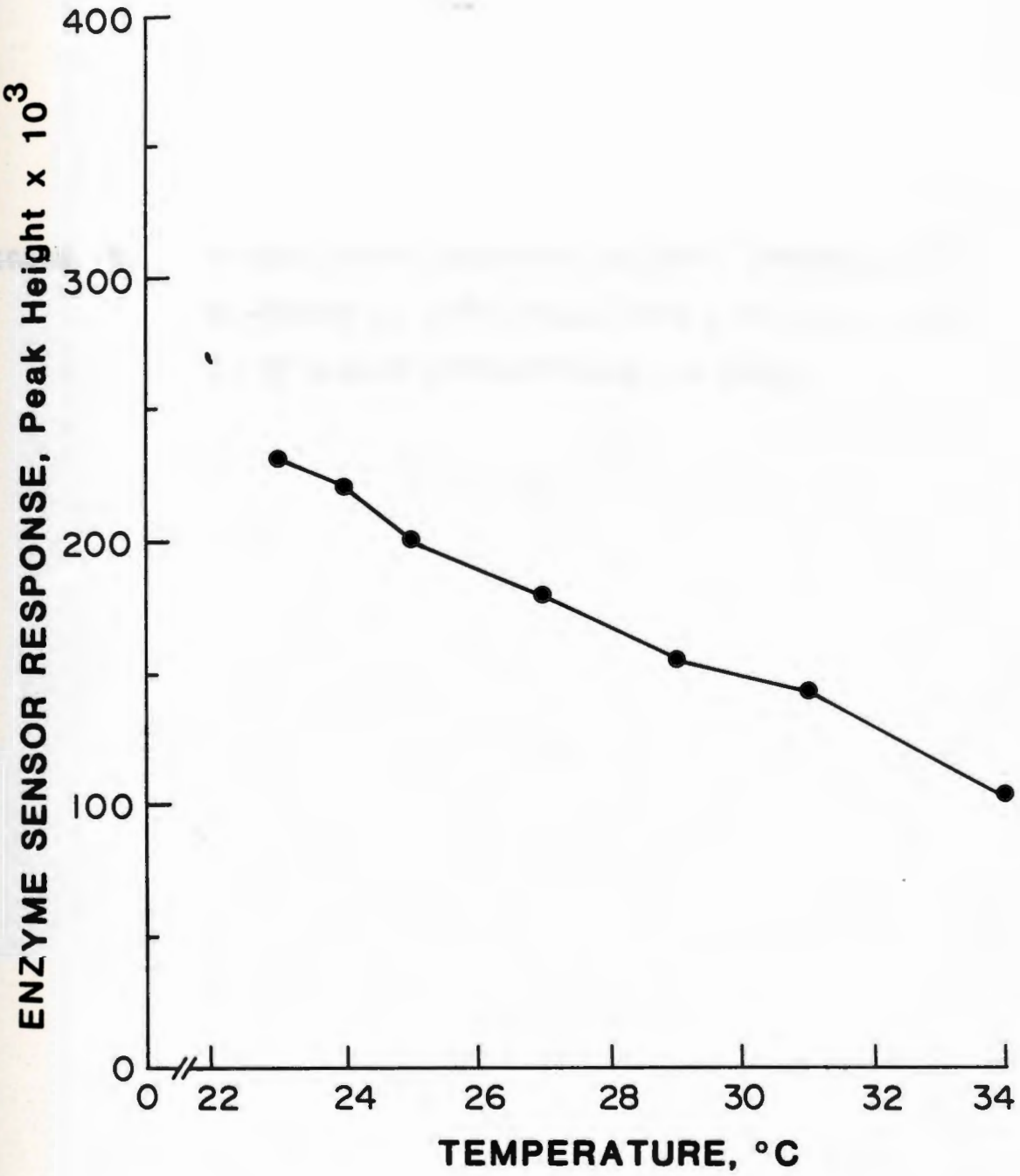
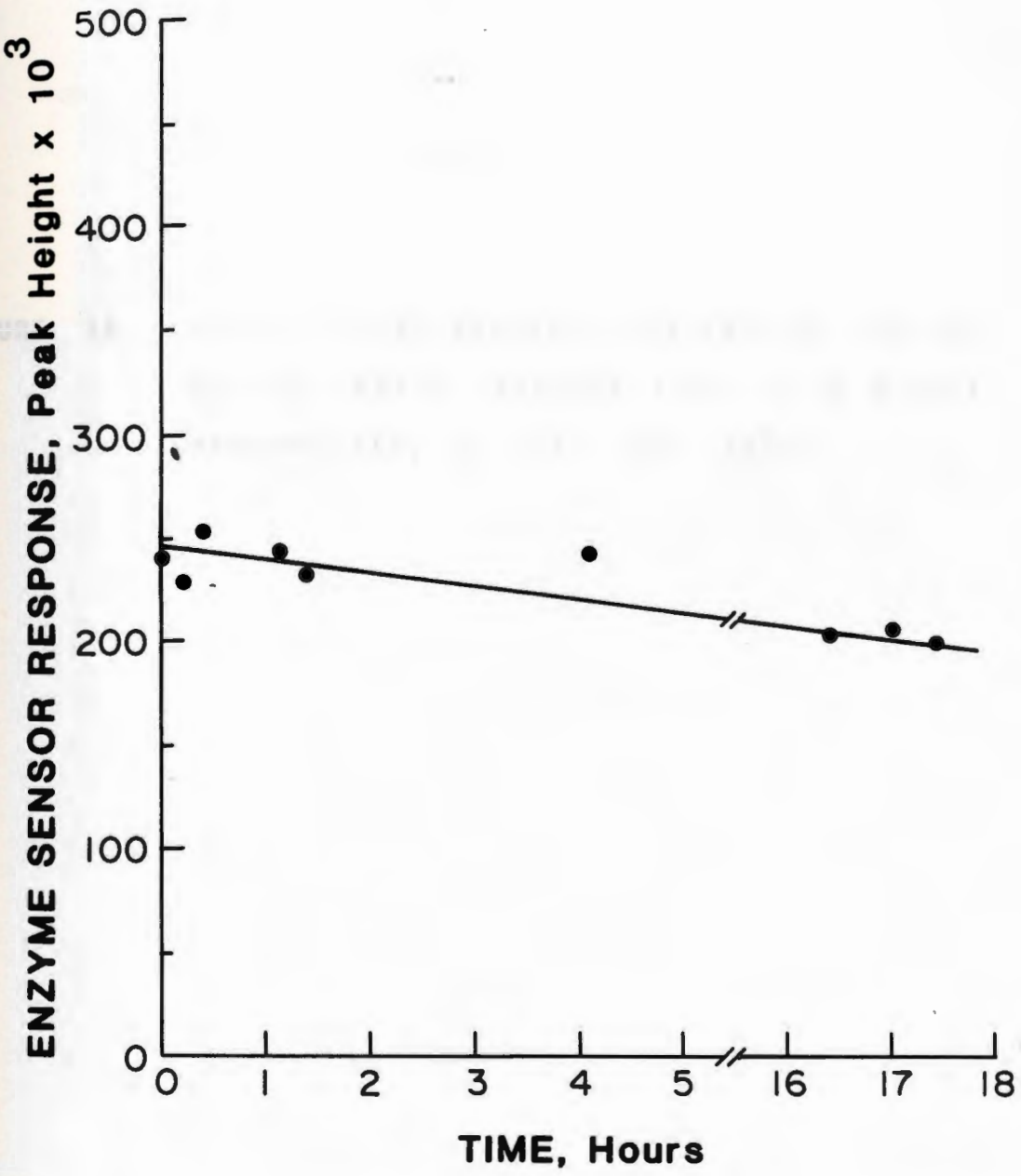

ENZYME SENSOR RESPONSE, Peak Height x 10^3
400
300
200
100
0
0
22
24
26
28
30
32
34
TEMPERATURE, °C

FIGURE 9. STABILITY OF XANTHINE OXIDASE IMMOBILIZED MEMBRANE AT 23^{0}c (FLOW RATE 1 ml/min., 100 ul OF 0.002% HYPOXANTHINE, pH 7.85).


ENZYME SENSOR RESPONSE, Peak Height x 10^3
500
400
300
200
100
0
0
1
2
3
4
5
16
17
18
TIME, Hours

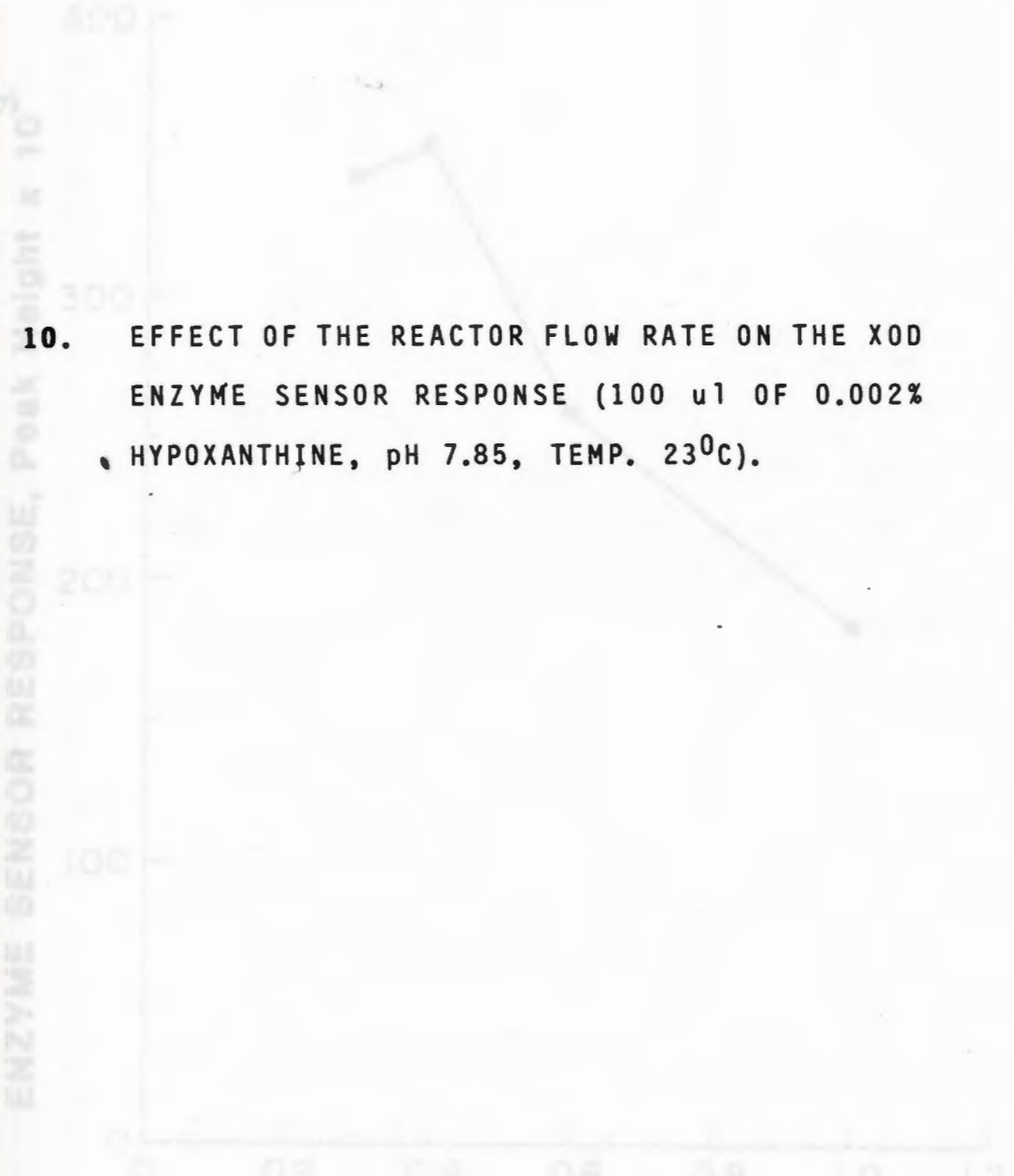
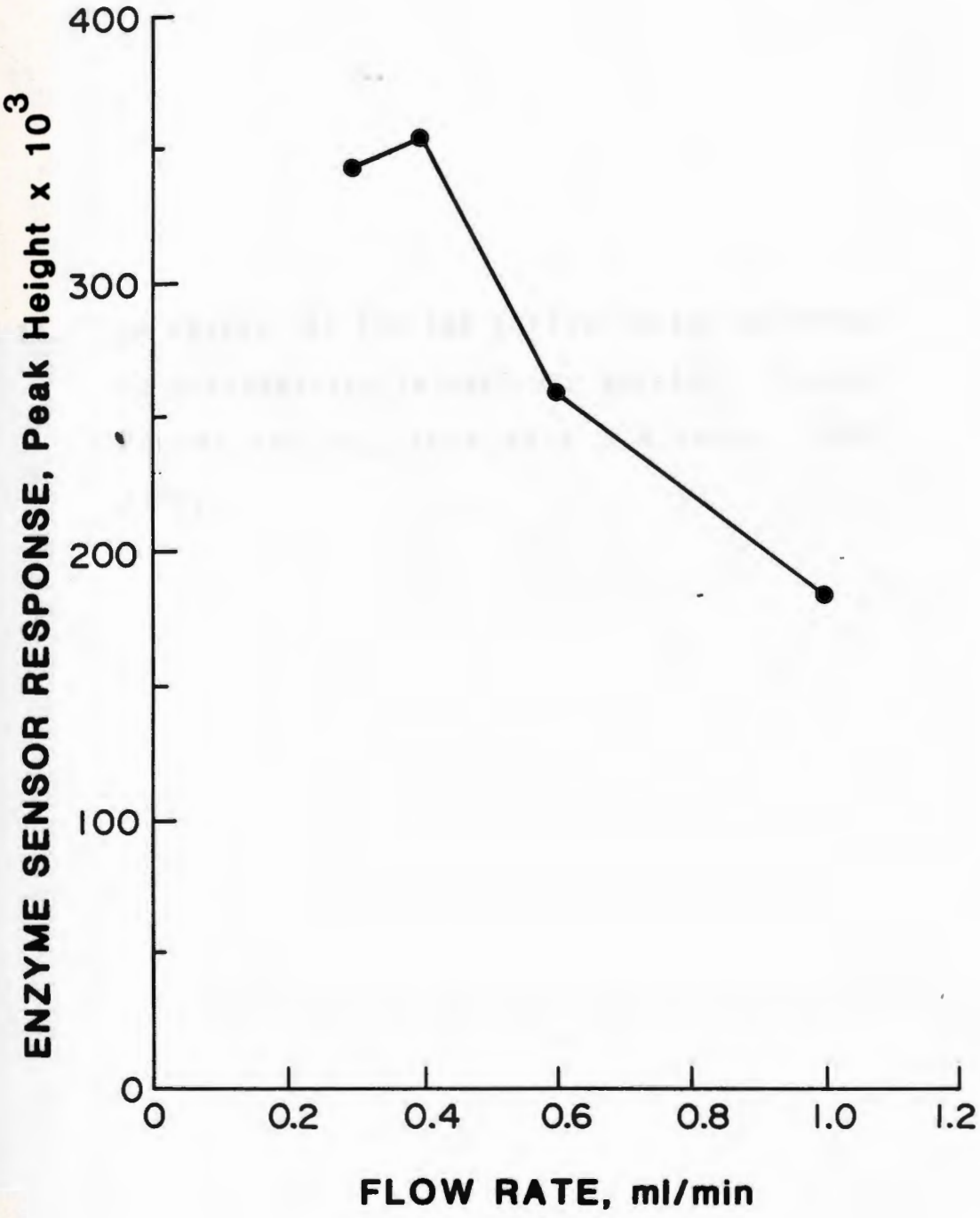


FIGURE 10. EFFECT OF THE REACTOR FLOW RATE ON THE XOD ENZYME SENSOR RESPONSE (100 ul OF 0.002% HYPOXANTHINE, pH 7.85, TEMP. 23^0C).


400
300
200
100
0
0
0.2
0.4
0.6
0.8
1.0
1.2
ENZYME SENSOR RESPONSE, Peak Height x 10^3
FLOW RATE, ml/min

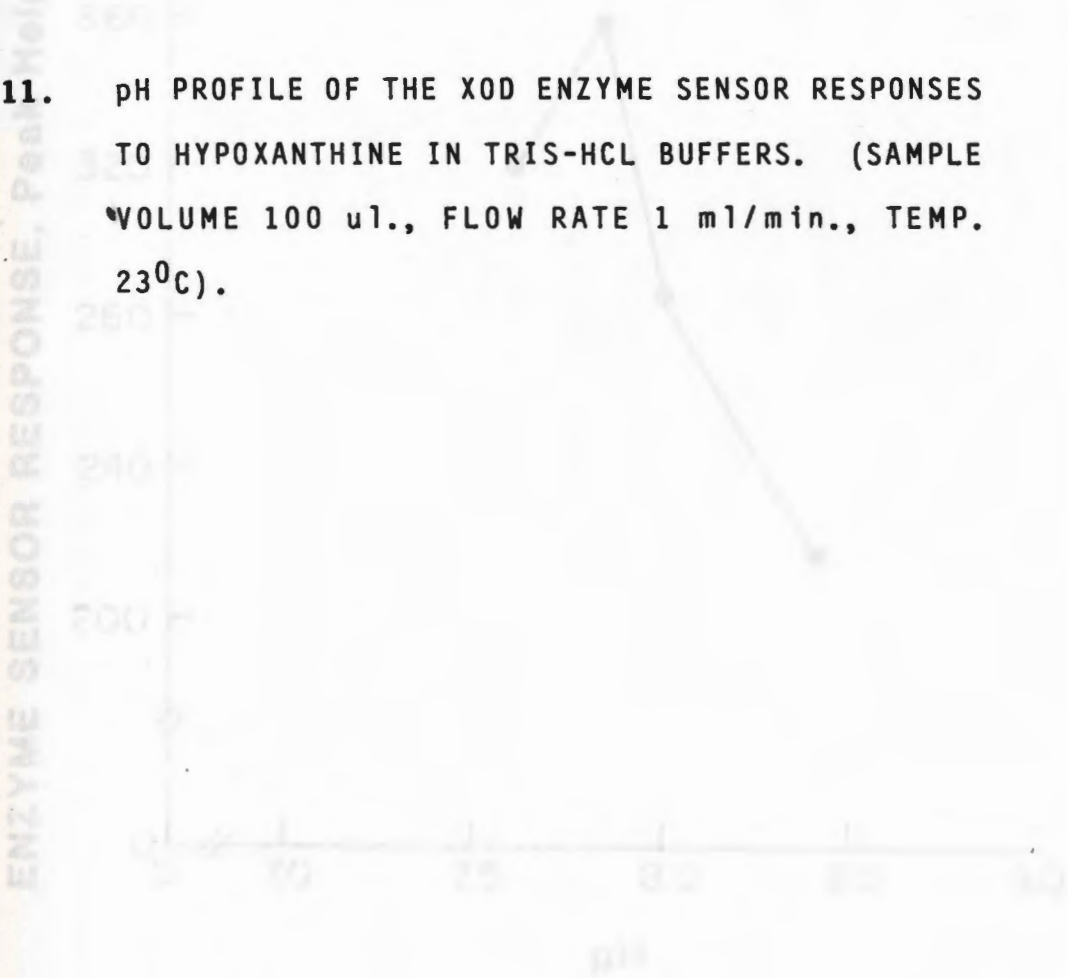
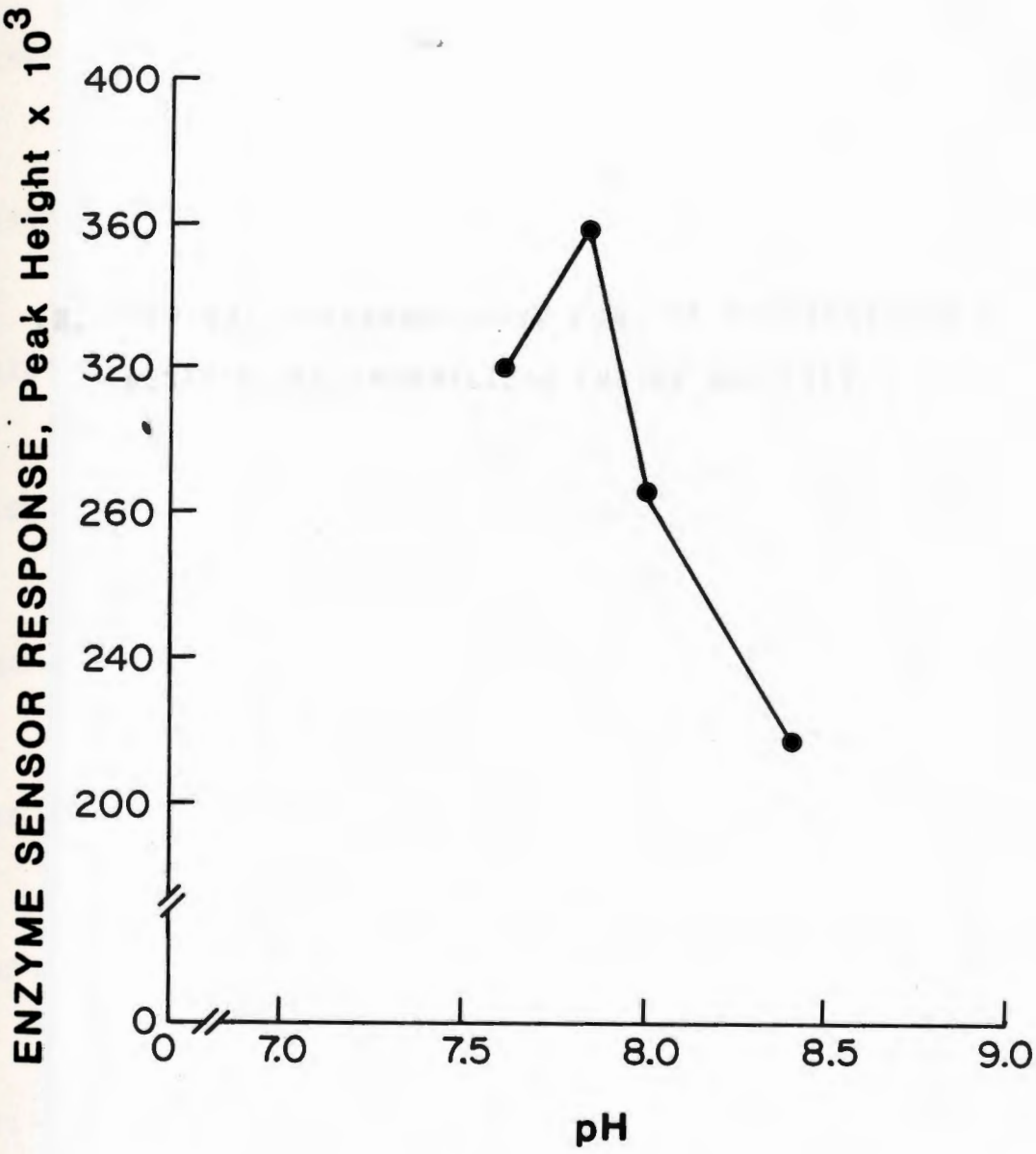


FIGURE 11. pH PROFILE OF THE XOD ENZYME SENSOR RESPONSES TO HYPOXANTHINE IN TRIS-HCL BUFFERS. (SAMPLE VOLUME 100 ul., FLOW RATE 1 ml/min., TEMP. $23^{0}C$).


ENZYME SENSOR RESPONSE, Peak Height x 10^3
400
360
320
260
240
200
0
0
7.0
7.5
8.0
8.5
9.0
pH

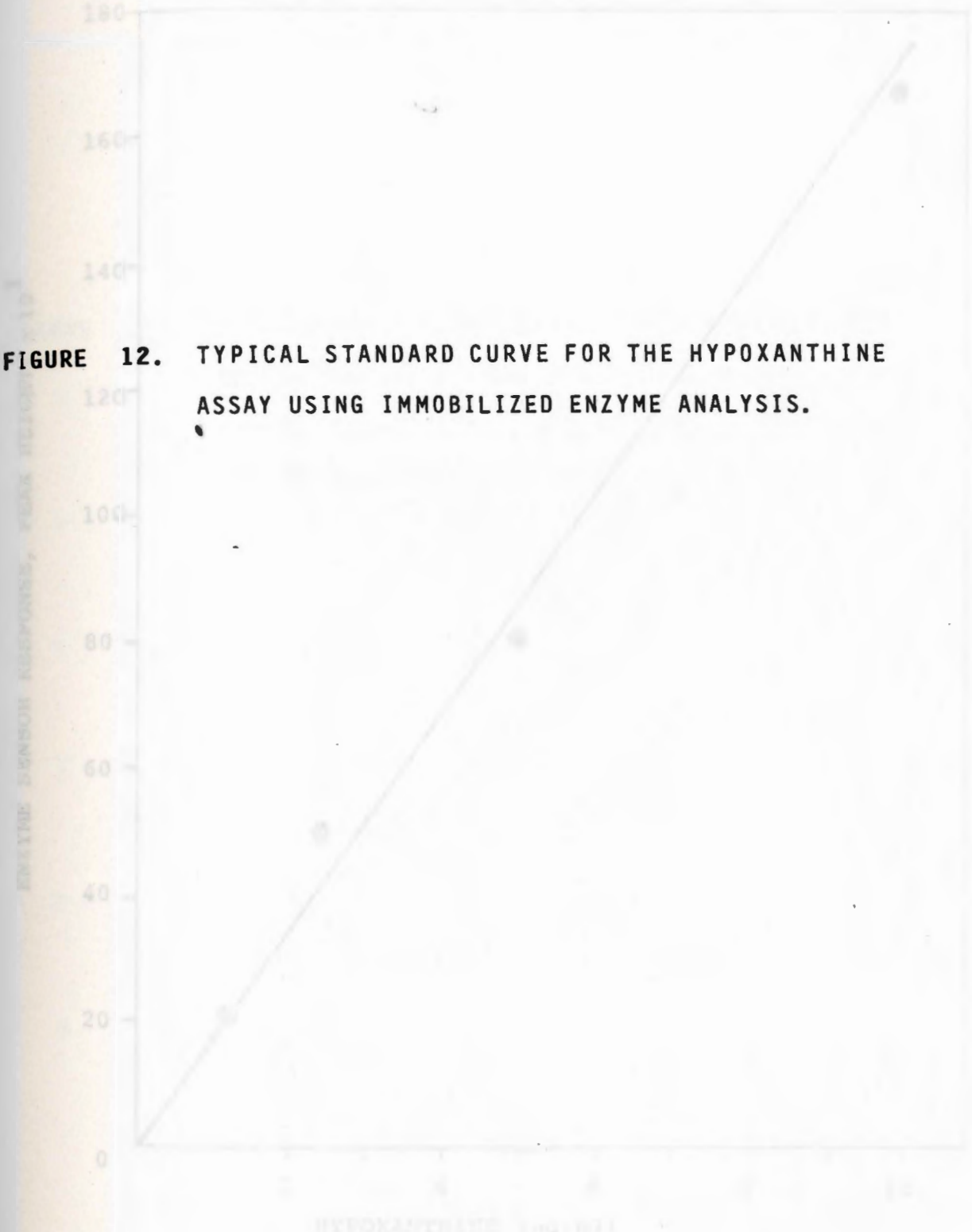
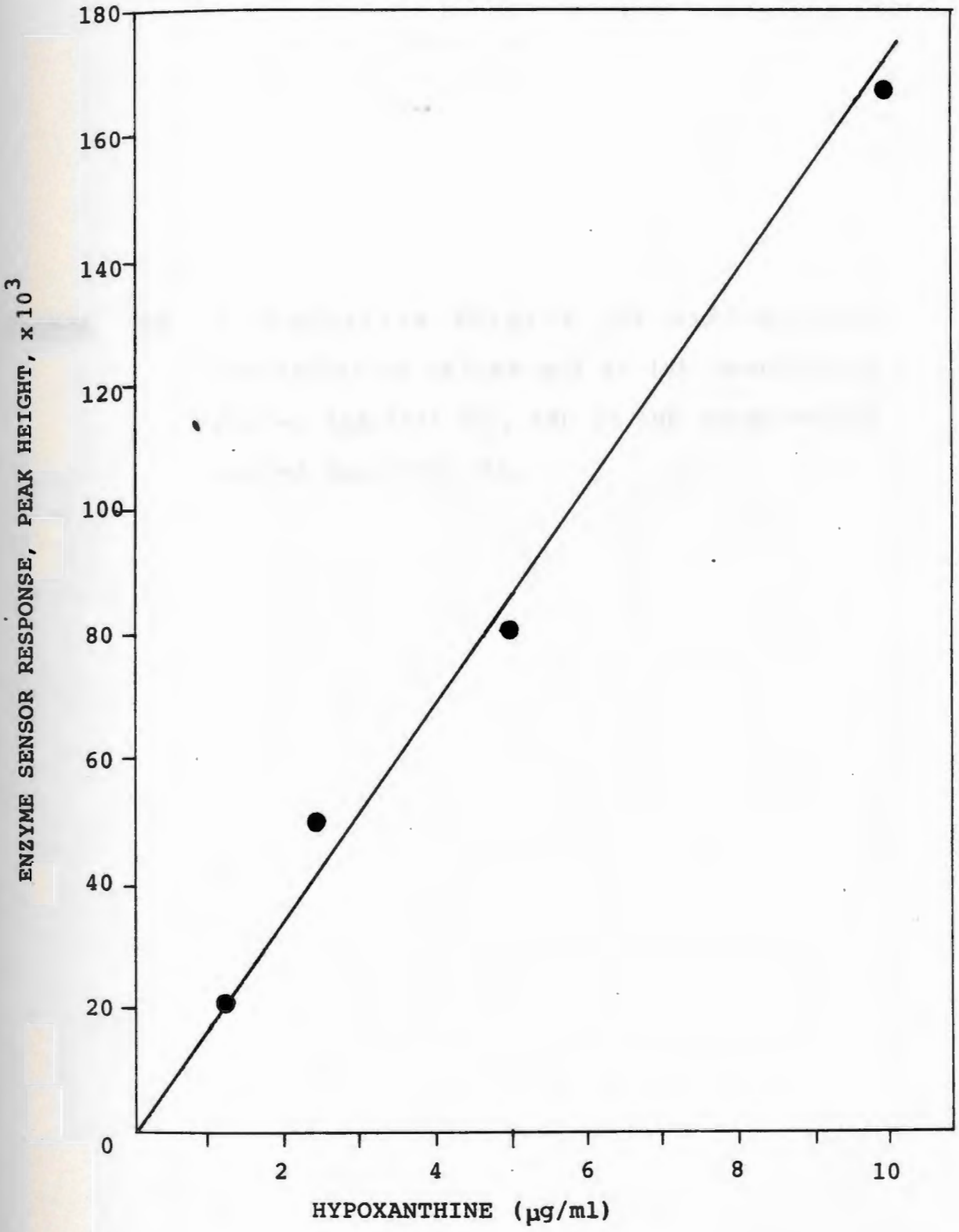


FIGURE 12. TYPICAL STANDARD CURVE FOR THE HYPOXANTHINE ASSAY USING IMMOBILIZED ENZYME ANALYSIS.


ENZYME SENSOR RESPONSE, PEAK HEIGHT, x 10^3
180
160
140
120
100
80
60
40
20
0
2
4
6
8
10
HYPOXANTHINE (µg/ml)

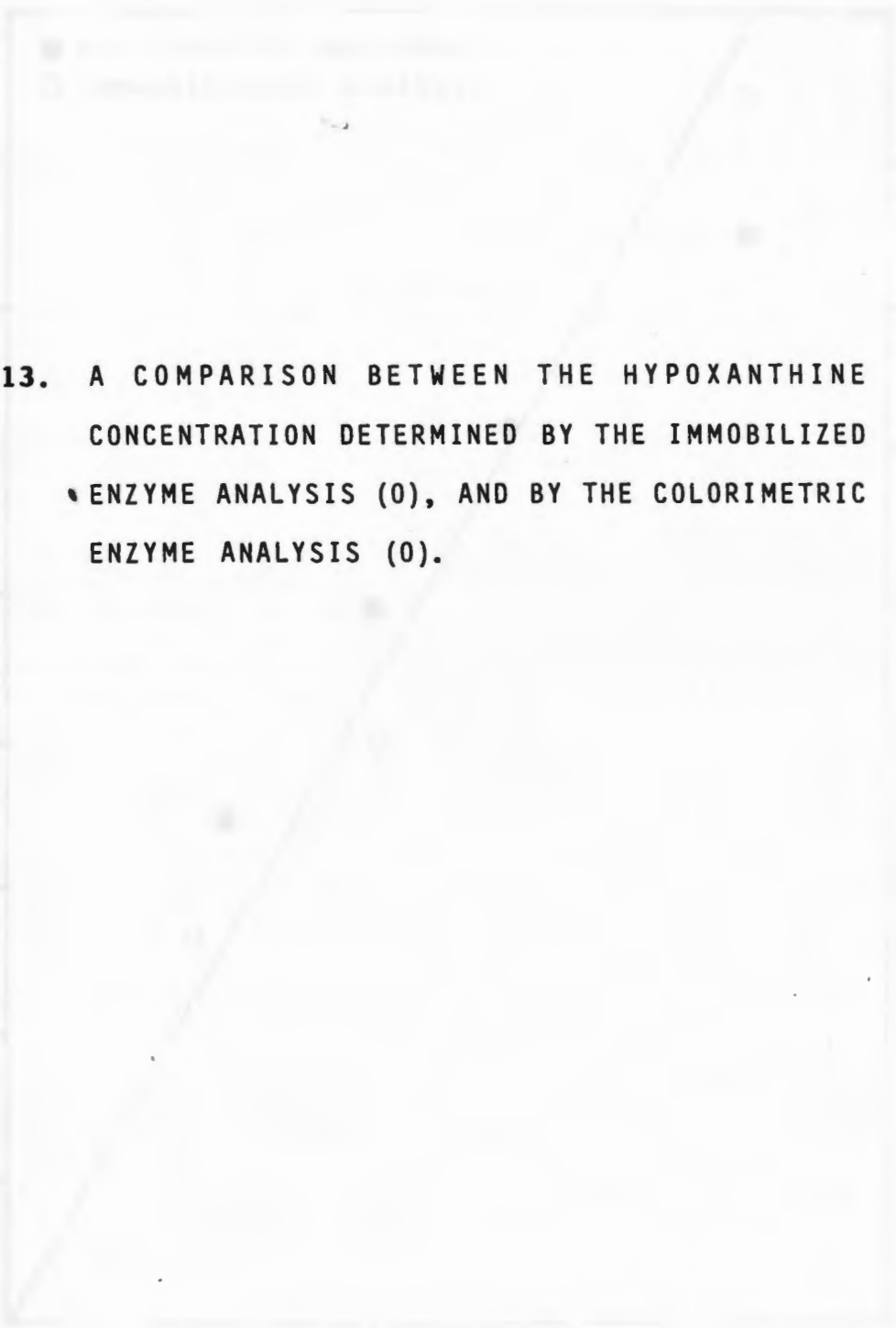
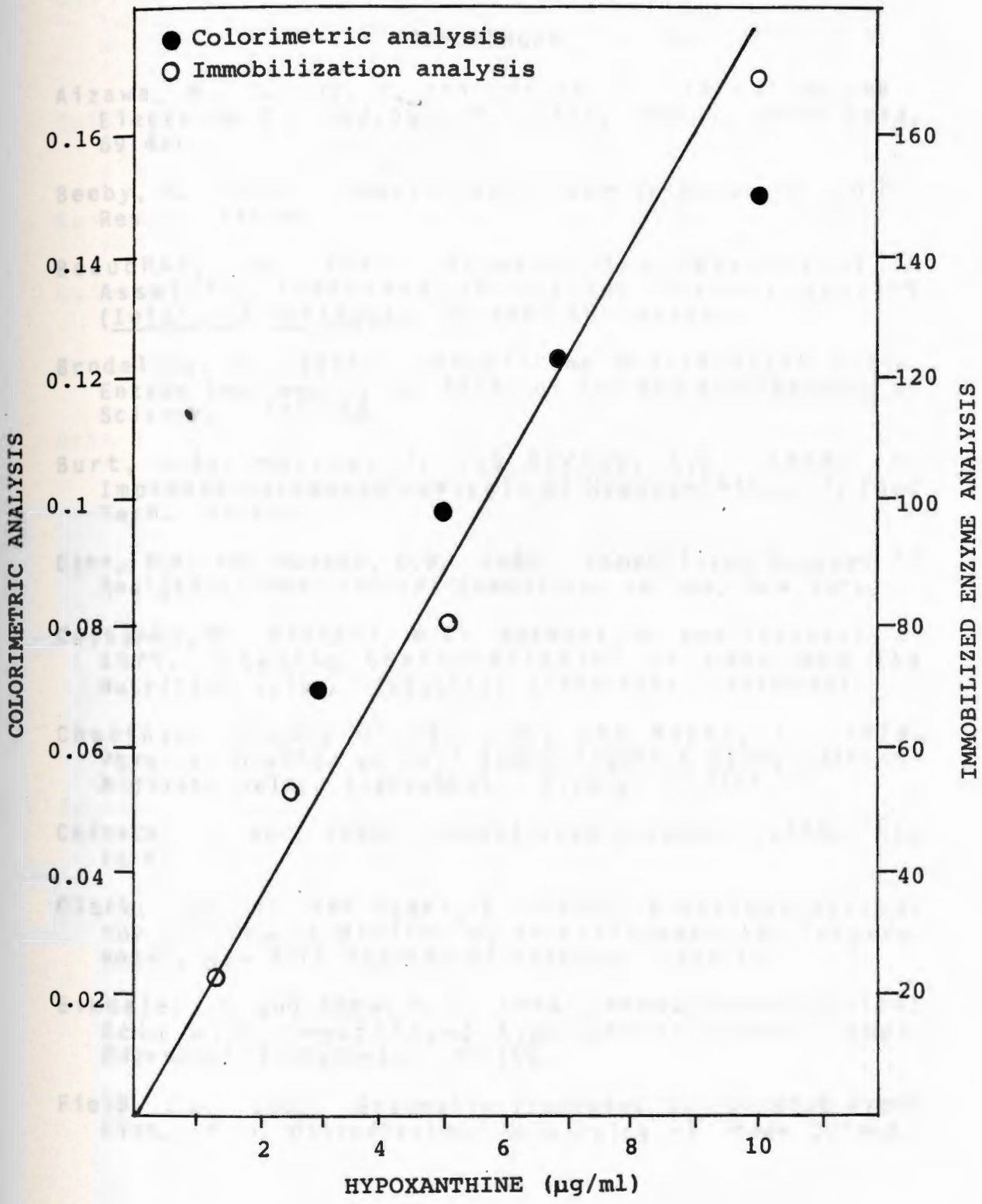


FIGURE 13. A COMPARISON BETWEEN THE HYPOXANTHINE CONCENTRATION DETERMINED BY THE IMMOBILIZED ENZYME ANALYSIS (O), AND BY THE COLORIMETRIC ENZYME ANALYSIS (O).


● Colorimetric analysis
○ Immobilization analysis
COLORIMETRIC ANALYSIS
IMMOBILIZED ENZYME ANALYSIS
HYPOXANTHINE (µg/ml)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
160
140
120
100
80
60
40
20
2
4
6
8
10

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MANUSCRIPT II

IMMOBILIZED XANTHINE OXIDASE MEMBRANE METHOD FOR THE MEASUREMENT OF HYPOXANTHINE

ABSTRACT

An immobilized xanthine oxidase analysis for hypoxanthine in fish, has been developed using a Ca-alginate membrane. Clark electrode, oxidase meter and continuous flow reactor. Fish (whiting) were stored in regular fresh water ice for 13 days, with two fish removed at approximately 48 hour intervals and frozen at -20°C for preservation and storage until analyzed. The fish tested showed that hypoxanthine accumulation occurred and increased in agreement with the standard colorimetric analysis, with increasing storage time until about 8 days and then decreased.

This analysis procedure was simple, rapid and may prove to be a useful procedure to assess fish freshness and as an alternative or adjunct to the more time consuming and expensive colorimetric analysis, which may also encourage the extension of this work to other food fish.

INTRODUCTION

An acceptable procedure to assess fish quality is necessary to protect the consumer and trader and to help the seafood industry with the manufacture of high quality products. Several methods and procedures have been developed, tried, and described to measure the quality of fish, such as volatile reducing substances, volatile nitrogen bases, ammonia (Farber and Ferro, 1956; Dugal, 1967), pH, Trimethylamine (Beatty and Gibbons, 1937), hypoxanthine and xanthine (Burt et al.; 1968, Beuchat, 1973; Jahns et al., 1976; Jones et al., 1964; Uchiyma, 1969; Jacober et al., 1981). These methods and procedures, however, require complicated operations, a long time for preparation, or are inaccurate, or costly; therefore, quick and simple methods are required.

Following the death of a fish, adenosine-5'-triphosphate (ATP) breaks down to the adenosine-5'-diphosphate and other related compounds, such as hypoxanthine and xanthine (Saito et al., 1959). Whereas, hypoxanthine or xanthine is accumulated with an increase of storage time and can be analyzed using xanthine oxidase.

Studies have shown hypoxanthine concentration to increase with increasing storage time and conclude that

hypoxanthine concentration is a useful index of the freshness assessment of fish (Spinelli, 1964; Beuchat, 1973; Jahns et al., 1976; Jacober et al., 1981; Collette, 1983).

An enzyme sensor specific for hypoxanthine in fish tissue was developed by Watanabe et al., 1983 using immobilized xanthine oxidase-membrane and an oxygen probe. The preparation of the immobilized enzyme membrane in that study was time-consuming and could result in loss of activity. The disadvantage of this method (covalent) were also described by Klibanov (1983) and others. In addition, the critical reagent 1, 8-di-amino-4-animomethyl octane, was not available in the United States and the only source was Japan (A Sahi Kasei Co.). Therefore, a rapid, simple, low cost, and routinely applicable procedure for immobilization would be preferable.

The Ca-alginate (entrapment method) meets these criteria (Klibanov, 1983; Kierstan, 1981; Brodelius, 1984; Al-Awfy, 1986).

The objective of this study was to measure the hypoxanthine in fish using the Ca-alginate-xanthine oxidase immobilized membrane developed by Al-Awfy (1986), when combined with the YSI Model 25 oxidase meter and YSI Clark 2510 electrode in a continuous flow reactor.

MATERIALS and METHODS

Materials

Reagents:

Xanthine oxidase [EC 1.2.3.3, from butter milk, grade 1 (50 units)], hypoxanthine were obtained from Sigma Chemical Corp., St. Louis, MO.; $CaCl_2$ from J. T. Baker Chemical Co., Philipsburg, N.J.; 50% glutaraldehyde was from Eastman Kodak Co., Rochester, N.Y.; and Kelco gel LV (specifically clarified low calcium alginate) was provided by Kelco Co., Clark, N.J. All other reagents used in this experiment were obtained from Fisher Scientific Company. Deionized distilled water was used throughout.

Equipment:

The YSI Model 25 oxidase meter and YSI-Clark 2510 electrode were obtained from Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio. The continuous flow reactor was designed in the Engineering Instrument Shop of the University of Rhode Island, Kingston, Rhode Island; the peristaltic pump was obtained from Buchler Instrument, Inc., Fort Lee, N.J. The reporting integrator 3390A was obtained from Hewlett Packard, Avondale, PA. and a septum injector was purchased from Rainin Instrument Co., Woburn, MA.

METHODS

Fish Storage and Handling

Trawl caught whiting (Merluccius bilanearis) were obtained at Point Judith, R.I., from the commercial day boat, Friesland, under the command of Captain Dykstra. The fish were gutted, headed, and washed carefully to remove blood, viscera residues, and the black lining of the peritoneal cavity. The fish were packed in an ice chest containing regular fresh water ice. The ice chest was stored at 4°C.

Sampling

On appropriate days, duplicate fish samples were removed from ice storage, filleted, skinned, packed in sterile plastic bags, and stored at -20°C for preservation and storage until analyzed.

Extraction

The frozen fish samples were extracted by blending 20g of the muscle in 60 ml of cold 1.0 N perchloric acid in a waring blender. The homogenates were filtered through Whatman filter paper #2, and stored at 2°C for hypoxanthine analyses.

Preparation of Ca-alginate Immobilized Enzyme Membrane

The Ca-alginate immobilized enzyme membrane was prepared as described by Al-Awfy (1986). Briefly, 1 g of Kelco gel LV (Algin) was added gradually to 50 ml distilled deionized water in a waring blender jar and blended at high speed until a uniform mixture was obtained. The blended mixture was stored at 4°C until needed. In a 0.5 cm depth and 4.5 cm diameter, flat dish, 2 ml of the 2% alginate solution and 46 ul of stock xanthine oxidase concentrate (21.74 u/ml) xanthine oxidase were mixed very well with a spatula. The mixture was sprayed with 5% $CaCl_2$ solution several times for about 10 minutes. Extra $CaCl_2$ was added to fill the dish and to cover the whole membrane. After that, the membrane was washed with distilled water and incubated in 4 ml. 0.05 M Tris-HCl buffer, pH 8.4, which contained 0.14 ml (0.0028%) 50% glutaraldehyde, (at 4°C) for 12 hours. The membrane was washed with distilled water and stored in 0.05 M Tris-HCl, PH 7.85, at 4°C until utilized.

Hypoxanthine Preparation for Analysis

A 5.0 ml aliquot of filtered perchloric acid fish extract was neutralized with 1.5 ml of 20% KOH and brought to a volume of 100 ml with 0.01 M Tris-HCl buffer, pH

7.85. Standard sample solutions consisting of 0 to 10 ug/ml hypoxanthine were prepared. These solutions were kept refrigerated (4°C) and warmed up to room temperature (23°C) before being used.

Immobilized XOD Assay Procedure

The continuous flow reactor (reaction chamber) was similar to the one described by Watanabe et al. (1983) with some modifications as reported by Al-Awfy (1986). A small circle of the immobilized membrane was cut by Pasteur pipet (about 0.7 cm diameter), attached to the oxidase probe, and covered with a dialysis membrane. A 0.05 M Tris-HCl buffer solution was transferred continuously to the reaction chamber by a peristaltic pump. Sufficient time was allowed for the probe to reach a steady state. Then 100 ul of a hypoxanthine standard or fish extraction solution was injected into the flow line. The maximum current measured as peak height by the HP-3309A integrator was directly proportional to the hypoxanthine concentration. Each assay was carreid out with at least duplicate injection, and the mean value was calculated. The temperature was controlled at 23°C during the assay.

Hypoxanthine Calculation

The hypoxanthine concentration was calculated and evaluated from the immobilized XOD procedure using two methods: A) from the standard curve, and B) by converting the peak height into mg using the following formula $\frac{\text{unknown}}{\text{reference}}$ x conc. of reference. (The unknown is the peak height reading of the examined sample; reference is the peak height of the standard solutin injected and concentration of reference is the concentration of the volume of the standard sample injected.)

Colorimetric XOD Analysis Procedure

Hypoxanthine in standards and fish extraction solutions was determined by the method of Jahns and Rand (1977), as modified by Jacober et al. (1981).

RESULTS AND DISCUSSION

Figure 1 and Table 1 show a comparison of hypoxanthine concentration in whiting during iced storage using the immobilized and colorimetric XOD analysis. The results show that the hypoxanthine concentration increased with increasing storage time until about 8 days and then decreased. At low substrate (hypoxanthine) concentrations the colorimetric analysis gave a lower reading than did the immobilized XOD probe. This may be due to enzyme inhibitors in the extract, or instrument sensitivity which may be more effected at low substrate concentrations. However, at high substrate concentrations, the values from both methods were similar.

The hypoxanthine concentration determined by the immobilized XOD probe was calculated and evaluated using two methods. The typical method used to calculate the concentration of an unknown compound, drawing a standard curve, and from that curve the concentration of the unknown was determined. This method was time consuming and liable to many errors regarding the construction of the standard curve, especially when drawing the line through the points. It was found that since peak height was used to measure the enzyme sensor response, it would

be easy to use the ratio formula method. As shown in Figure 1 and Table 1 both methods gave hypoxanthine concentration values which were close to each other. It was found that using the ratio formula was very quick, simple, and less time consuming, and more applicable for routine work.

A significant finding of this study was that the results of the immobilized enzyme analysis resembled other studies which have been reported to assess the measurement of the freshness of fish using hypoxanthine as index of freshness (Collette and Rand, 1983; Jahns et al., 1976; Jacober et al., 1981; Beuchat, 1973; Spinelli et al., 1964; Shewan and Jones, 1957). Hypoxanthine accumulated in the fish muscle post-harvest to a peak value (8 days for Whiting), or about the point of incipient spoilage. Then the hypoxanthine exhibited the usual pattern of decline as the fish entered the spoilage phase and became unacceptable.

CONCLUSION

The immobilized enzyme analysis to measure hypoxanthine in fish presented in this paper was rapid, simple to perform, and less time-consuming, and could be used for routine work. This analysis procedure may prove to be a useful procedure to assess fish freshness and as an alternative or adjunct to the more time consuming colorimetric analysis.

Table 1 Comparison of hypoxanthine concentration in fish using immobilized enzyme analysis and colorimetric analysis.

Days ice storage	Hypoxanthine *		
	Immobilization		Colorimetric
	using formula	standard curve	
1	18.15	14.04	6.5
4	19.52	19.81	9.7
6	23.03	22.4	16.3
8	29.24	29.24	25.8
11	25.14	25.71	22.3
13	21.67	21.92	20.6

* hypoxanthine in Mg/ml

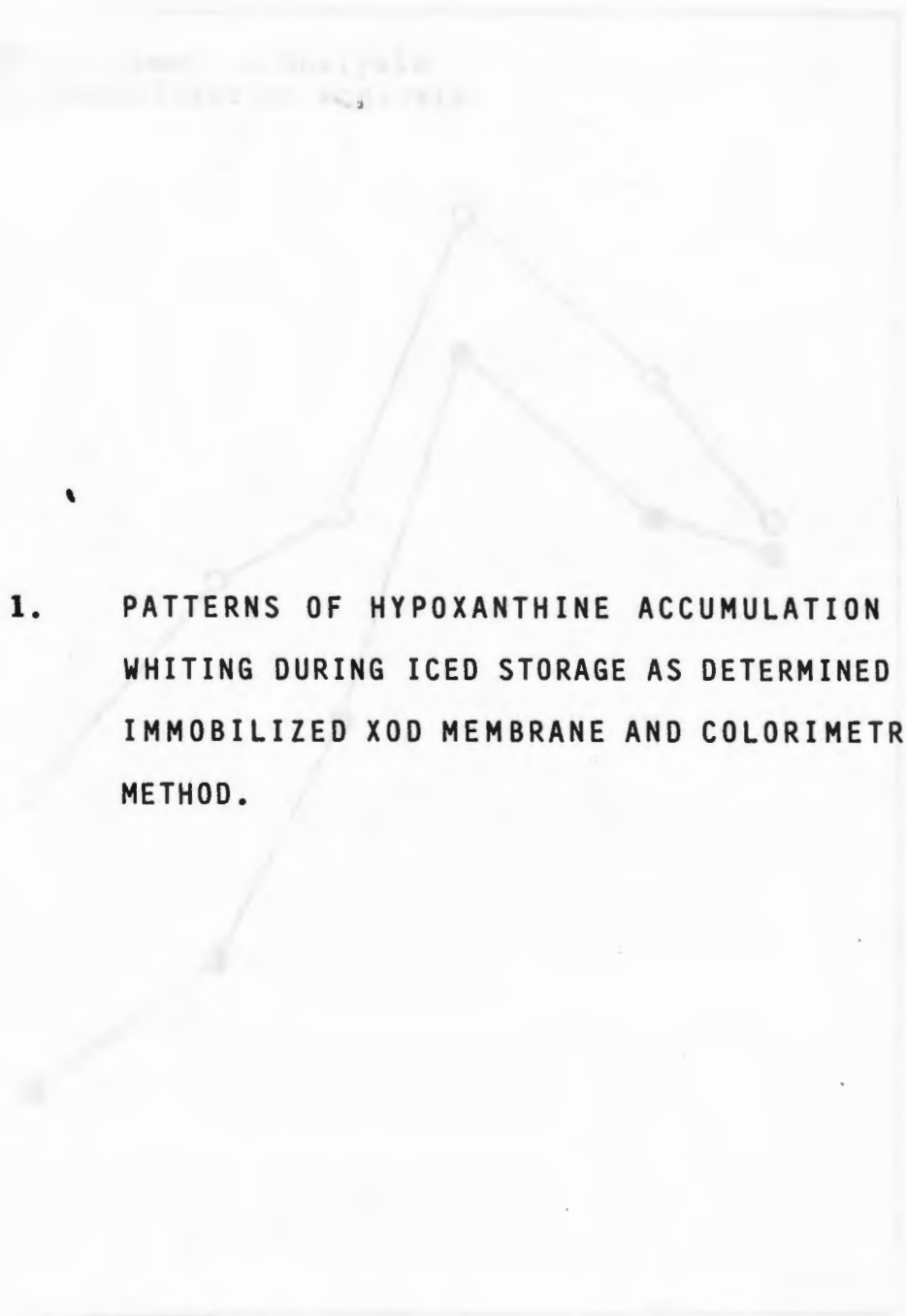
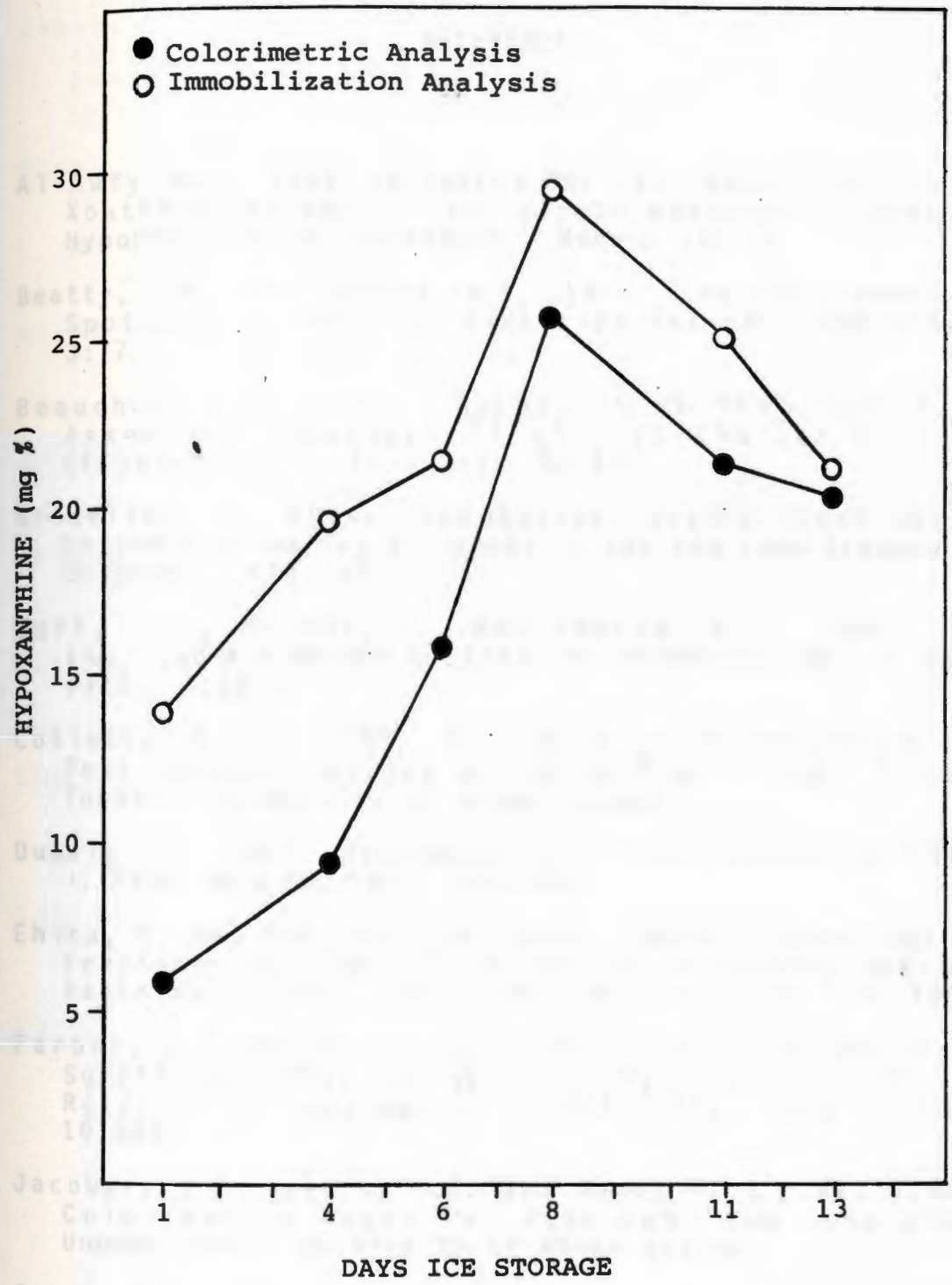


FIGURE 1. PATTERNS OF HYPOXANTHINE ACCUMULATION IN WHITING DURING ICED STORAGE AS DETERMINED BY IMMOBILIZED XOD MEMBRANE AND COLORIMETRIC METHOD.


Colorimetric Analysis
Immobilization Analysis
HYPOXANTHINE (mg %)
30
25
20
15
10
5
1
4
6
8
11
13
DAYS ICE STORAGE

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MANUSCRIPT III

IMMOBILIZED XANTHINE OXIDASE MEMBRANE METHOD FOR THE MEASUREMENT OF HYPOXANTHINE AND THE ASSESSMENT OF MEAT QUALITY

ABSTRACT

An immobilized xanthine oxidase probe for hypoxanthine analysis has been developed using a Ca-alginate membrane, a Clark electrode, oxidase meter and a continuous flow reactor. fresh Meats (beef and lamb) were stored in a 2-4^{o}C cold room. On appropriate days, quadruplicate pieces of meat samples were cut for sensory evaluation. After sensory evaluation, the samples were frozen at -20^{o}C for preservation and storage until analyzed for hypoxanthine. Sensory evaluation results, especially the edibility index, indicated that until day 7 the majority of potential consumers would purchase and cook the given sample of meats, which coincided with a low hypoxanthine concentration. However, after day 7, the acceptability declined and the hypoxanthine concentration increased. This analysis procedure was simple, rapid, and may prove to be a useful procedure to assess meat freshness.

INTRODUCTION

Food quality as a fresh, desirable produced with high consumer acceptance is an important factor especially in the Third World countries who import much of their food. The meat shipped to those countries either frozen or in cold storage, is a food product which is particularly susceptible to temperature fluctuations during shipment, resulting in deterioration of the meat and poor consumer acceptance. Therefore, a rapid, inexpensive, and acceptable procedure is necessary to assess imported meat quality, to protect the consumer and trader, and to help ensure that food industries ship only high quality products to these Third World countries. Several methods and procedures have been developed to measure the quality of meat, such as extract-release volume (Jay, 1964), pH (Shelef and Jay, 1970; Swift et al., 1960); monoamines (Karube, 1980); color (Strange et al., 1974), thiabarbituric acid (Witte et al., 1970), total volatile nitrogen and tyrosin (Pearson, 1968a), free fatty acid levels (Vasundhara, 1983), and hypoxanthine, xanthine and inosine monophosphate (Parris et al., 1983; Pizzacaro, 1978; Cattaneo, 1979; Platz et al., 1978). Most of these

and other methods and procedures were reviewed by Pearson (1968b), Strange et al. (1977), and Gil (1983). Most of these methods and procedures required complicated operations, long preparation time or were inaccurate and/or costly; therefore, quick, simple and reliable methods still must be developed.

Following the death of the animal, adenosine-5'-triphosphate (ATP) breaks down to the adenosine-5'-diphosphate and other related compounds, such as hypoxanthine and xanthine (Tsai et al., 1972, Price and Schweigert, 1970, Lawrie, 1979). Because hypoxanthine or xanthine accumulates with an increase of storage time, these compounds can be analyzed using xanthine oxidas (XOD). Studies have shown that hypoxanthine concentration is a useful index of the freshness assessment of meat (Parris et al., 1983; Pizzacaro, 1978; Cattaneo, 1979; Platz et al., 1978).

Recently, a biological electrode combined with an immobilized membrane has been developed and proven to be a useful method for measuring amines in meat (Karube et al., 1980). A biological electrode for analysis of hypoxanthine in fish has recently been developed (Watanabe et al., 1983; Al-Awfy, 1986). However, at this time there have been no reports on an enzyme sensor specific for

measuring hypoxanthine in meat.

The objective of this study was to measure the hypoxanthine in meat using the Ca-alginate-xanthine oxidase immobilized membrane developed by Al-Awfy (1986) combined with the YSI Model 25 oxidase meter and YSI Clark 2510 electrode in a continuous flow reactor.

MATERIALS and METHODS

Materials

Reagents:

Xanthine oxidase [EC 1.2.3.3, from butter milk, grade 1 (50 units)] and hypoxanthine (6-hydr-oxypurine) were obtained from Sigma Chemical Corp., St. Louis, MO.; anhydrous, purified calcium chloride was purchased from J. T. Baker Chemical Co., Philipsburg, N.J.; 50% glutaraldehyde was from Eastman Kodak Co., Rochester, N.Y.; and Kelco gel LV (specifically clarified low calcium alginate) was provided by Kelco Co., Clark, N.J. All other materials used in this experiment were reagent grade and obtained from Fisher Scientific Company. Deionized distilled water was used throughout.

Equipment:

The YSI Model 25 oxidase meter and YSI-Clark 2510 electrode were obtained from Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio. The continuous flow reactor was designed in the Engineering Instrument Shop of the University of Rhode Island, Kingston, Rhode Island; the peristaltic pump was obtained from Buchler Instrument, Inc., Fort Lee, N.J. The reporting integrator 3390A was obtained from Hewlett Packard, Avondale, PA. and a septum injector was purchased from Rainin Instrument Co., Woburn, MA.

METHODS

Meat Storage and Handling

Fresh beef and lamb meat were obtained from local slaughter houses in Rhode Island. The meats were collected (from the slaughter houses) on the day of carcass slaughter, sealed in plastic bags and stored in a 2-4°C cold room.

Sampling

On appropriate days, quadruplicate pieces of meat samples were cut for sensory evaluation. After sensory evaluation, the samples were packed in separate sterile plastic bags and stored at -20°C for preservation and storage until analyzed for hypoxanthine.

Sensory evaluation

On sampling day, pieces of meat samples were presented in well iced, coded stainless steel trays to a panel of at least 8 graduate students (untrained) for raw sensory evaluation based on odor, color and appearance, and edibility. The raw odor score scale as decribed by Pearson (1968) was used, and the scaling system of

Preparation of Ca-alginate Immobilized XOD Membrane

The Ca-alginate immobilized enzyme membrane was prepared as described by Al-Awfy (1986). Briefly, 1 g of Kelco gel LV (Algin) was added gradually to 50 ml distilled deionized water in a Waring blendor jar. The mixture was blended at high speed until a uniform solution was obtained. The blended mixture was stored at 4°C until needed. In a 0.5 cm depth and 4.5 cm diameter flat dish, 2 ml of the 2% alginate solution and 46 ul of stock xanthine oxidase concentrate (21.74 u/ml) were mixed very well with a spatula. Then the mixture was sprayed with 5% $Cacl_2$ solution several times for about 10 minutes. Extra $Cacl_2$ was added to fill the dish and cover the whole membrane. After that, the membrane was washed with distilled water and incubated in 4 ml. 0.05 M Tris-HCl buffer, pH 8.4, which contained 0.14 ml (0.0028%) 50% glutaraldehyde, for 12 hours. Then the membrane was washed with distilled water and stored in 0.05 M Tris-HCl, pH 7.85, at 4°C until utilized.

Immobilized Enzyme Hypoxanthine Assay

The continuous flow reactor was similar to the one described by Watanabe et al. (1983) with some modification

as reported by Al-Awfy (1986). A small 0.7 diameter circle of the immobilized membrane was cut by Pasteur pipet, attached to the oxidase probe, and covered with dialysis membrane. A 0.05 M Tris-HCl buffer solution was transferred continuously to the reaction chamber by the peristaltic pump. Sufficient time was allowed for the probe the stabilize, which indicated when a steady state was reached. Then 100 ul of a hypoxanthine standard or fish extraction solution was injected into the flow line. The maximum current measured as peak height by the HP-3309A integrator, was directly proportional to the hypoxanthine concentration. Each assay was carried out with at least duplicate injections, and the main value was calculated. The temperature was controlled at 23°C during the assay.

Hypoxanthin Calculation

The hypoxanthine concentration was calculated as described by Al-Awfy (1986).

RESULTS AND DISCUSSION

Raw beef and lamb stored in a cold room were evaluated for their odor, color and appearance, and edibility. Figures 2 and 3 show the panel scores for odor and color and appearance for beef and lamb. The results show that on the first evaluation after 4 to 5 days of storage at 2-4°C, all the panelists agreed that the meats had a fresh odor and very good color and appearance. With subsequent determinations, the scores decreased proportionally with an increase in storage time. With lamb, at day 10 the odor was still acceptable and color and appearance were still slightly good. After 12 days, the odor of lamb meat was unacceptable and color and appearance gave a borderline score indicating the product had reached the limit of consumer acceptability. The beef product remaines acceptable in odor and color/appearance until day 13. At day 15 the color and appearance of meat had become unacceptable. By day 18, both the odor and color and appearance scores had exceeded the limit of consumer acceptability.

The edibility was studied to see if the consumer would purchase, cook and eat a given sample of these meats, and the edibility index, which represents the

decision of the consumer, was calculated and compared with odor classes as seen in Tables 1 and 2. Meats exhibiting a fresh odor, had an edibility index of 1.0, and that indicated that the meats would be acceptable to virtually 100% of potential consumers. But with increasing storage time, the edibility index decreased. Lamb meat, by day 7, when its odor was acceptable would be bought and eaten by 88% of the consumers; this declined to 45% at day 10. By day 12, when just spoiled, nobody would buy or accept this meat; whereas with beef, by day 8, when its odor was acceptable about 75% of the consumers would buy and eat this meat, but this dropped to 50% by day 13 and then to 16% when its odor was unacceptable.

The hypoxanthine concentration in beef and lamb meat was studied and evaluated using both the immobilized enzyme analysis and colorimetric analysis. Figures 4 and 5 compare the analysis of hypoxanthine concentration in lamb and beef meat on different days of refrigerated storage by both methods. The results showed that the hypoxanthine accumulation occurred with increasing storage time and both methods produced the same pattern but the colorimetric method was always lower. At low hypoxanthine concentrations the colorimetric analysis gave a lower reading than did the immobilizing analysis. This may be

due to the same reasons which were explained in Manuscript II. As seen in Figure 4, the hypoxanthine concentration in lamb meat in the first 7 days of cold storage was less than 7 mg% by both methods. At day 10 when the lamb had marginal sensory characteristics, the hypoxanthine had started to increase; and then increased sharply to more than 20 mg% by day 12 by both methods when the product was no longer acceptable. Figure 5 shows that the hypoxanthine concentration in beef meat in the first 11-13 days of storage increased steadily to about 14-17 mg%. The hypoxanthine level in beef remained in this range until storage day 18. At day 20, following sensory determination of spoilage onset, there was some indication of a concentration decline.

The typical method used to calculate the hypoxanthine concentration of an unknown solution was by drawing a standard curve, and from that curve the concentration of the unknown was determined. This method was time consuming and liable to many errors regarding of the construction the standard curve, especially when drawing the line through the points.

The hypoxanthine concentration determined by immobilized XOD analysis was calculated and evaluated using two methods: A) from the standard curve, and B)

using the formula $\frac{\text{unknown}}{\text{reference}}$ x conc. of reference.

Since peak height was used to measure the enzyme sensor response, it was though that method B (formula) would be easy to use. As shown in Table 3 and 4, good agreement was obtained between the values determined by both methods. It was concluded that using method B was very quick, simple, and less time consuming, and more applicable for routine work.

These results show that the sensory evaluation results, especially the edibility index, indicated that through day 7 the majority of potential consumers would purchase and cook the given sample of lamb and through day 11, the given sample of beef. Generally, these were meat samples in which the hypoxanthine concentration was below about 14 mg%. After these key points in storage, the acceptability declined for the majority of consumers, and the hypoxanthine concentration increased dramatically. This relationship between the consumer's sensory evaluation results and hypoxanthine accumulation provided a consistent index of quality. Generally, when consumer acceptability declined, the hypoxanthine concentration exceeded 14 mg%. Therefore, determining hypoxanthine concentration by the procedure presented in this study

would be effective method for the assessment of meat quality.

Comparison of this study with previous workers (Pizzacaro, 1978; Cattaneo, 1979) provides further evidence that hypoxanthine could reflect sensory evaluation and can be used as a freshness index.

The significance of this study was using immobilized enzyme analysis may prove to be a useful procedure to assess meat freshness and as an alternative or adjunct to the more time consuming and expensive colorimetric analysis, which also encourages extension of this work to toher meats and meat products.

CONCLUSION

The immobilized enzyme analysis to measure hypoxanthine in meats presented in this paper was rapid, simple to perform, and less time-consuming, than the usual colorimetric method. The results appeared to indicate better sensitivity, since the analysis of identical samples always gave higher concentration, of hypoxanthine. In addition, there was remarkable agreement with sensory evalaution of meat, indicating the possibility of establishing a hypoxanthine level in meat for quality determination.

Table 1. Edibility index, odor and organoleptic classes of raw lamb meat held in cold storage between 2-4°C for 14 days.

Days Storage In Cold Room	Edibility Index	Raw Meat Odor	Organoleptic Class
4	1.0	Fresh	9
7	0.88	Acceptable	7
10	0.45	Acceptable	7
12	0.0	Just Spoiled	5
14	0.0	Well Spoiled	3

Table 2. Edibility index, odor and organoleptic classes of raw beef meat held in cold storage between 2-4°C for 20 days.

Days storage In Cold Room	Edibility Index	Raw Meat Odor	Organoleptic Class
5	1.0	Fresh	9
8	0.75	Acceptable	7
11	0.56	Acceptable	7
13	0.5	Just Acceptable	6
15	0.22	Just Acceptable	6
18	0.25	Just Spoiled	5
20	0.16	Unacceptable	4

Table 3 Comparison of hypoxanthine concentration using immobilized enzyme analysis and colorimetric analysis for lamb meat.

Days storage in cold room	Hypoxanthine *		
	Immobilization		Colorimetric
	using formula	standard curve	
4	5.6	6.4	1.6
7	5.9	6.8	6
10	12.8	14.0	7.5
12	23.8	25.44	18.4
14	25.6	28.3	21.44

* hypoxanthine in mg %

Table 4 Comparison of hypoxanthine concentration using immobilized enzyme analysis and colorimetric analysis for beef meat.

Days storage in cold room	Hypoxanthine *		
	Immobilization		Colorimetric
	using formula	standard curve	
5	8.32	9.44	2.0
8	9.6	10.41	10
11	14.92	16.64	12
13	14.83	16.48	13
15	13.42	15.04	12.8
18	15.48	17.2	14.8
20	12.74	14.93	12.72

* hypoxanthine in mg %.

FIGURE 1. THE EVALUATION SHEET USED BY SENSORY PANELISTS TO DETERMINE THE ODOR, COLOR AND APPEARANCE AND ACCCEPTABILITY OF RAW MEAT.

SENSORY EVANULATION OF RAW MEAT

NAME: _____ SAMPLE: _____

DATE: _____

Please carefully read the following descriptions and evaluate these samples. Circle the appropriate description to show your evaluation.

A - Raw odor	B - Color & Appearance
very fresh	excellent
fresh	very good
fairly fresh	good
acceptable	slightly good
just acceptable	borderline plus
just spoiled	borderline
unacceptable	borderline minus
well spoiled	slightly poor
nearly putrid	poor
putrid	extremely poor

C - Acceptability

Would you purchase, cook and eat this meat?

_____ YES _____ NO

Comments:

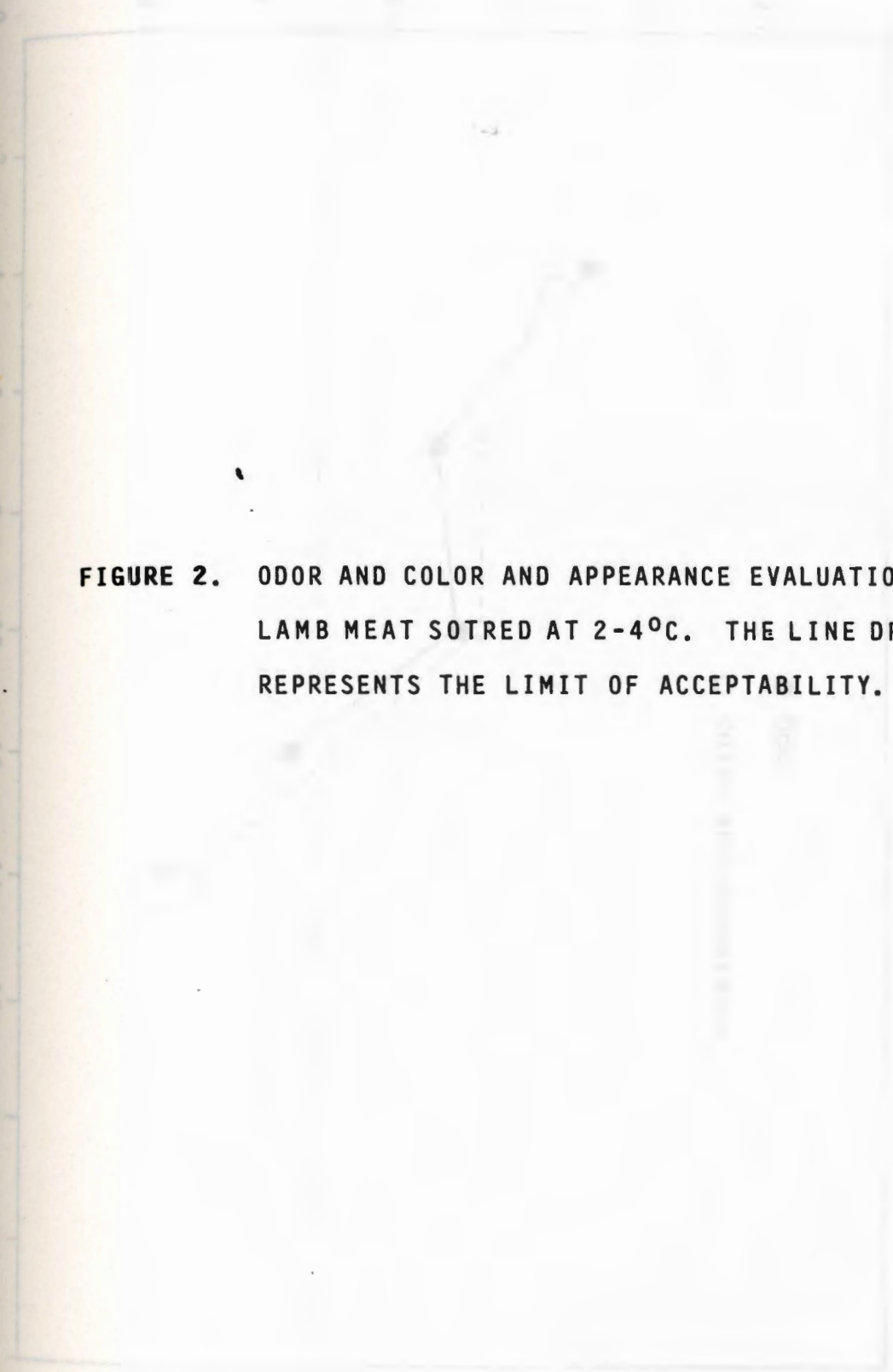
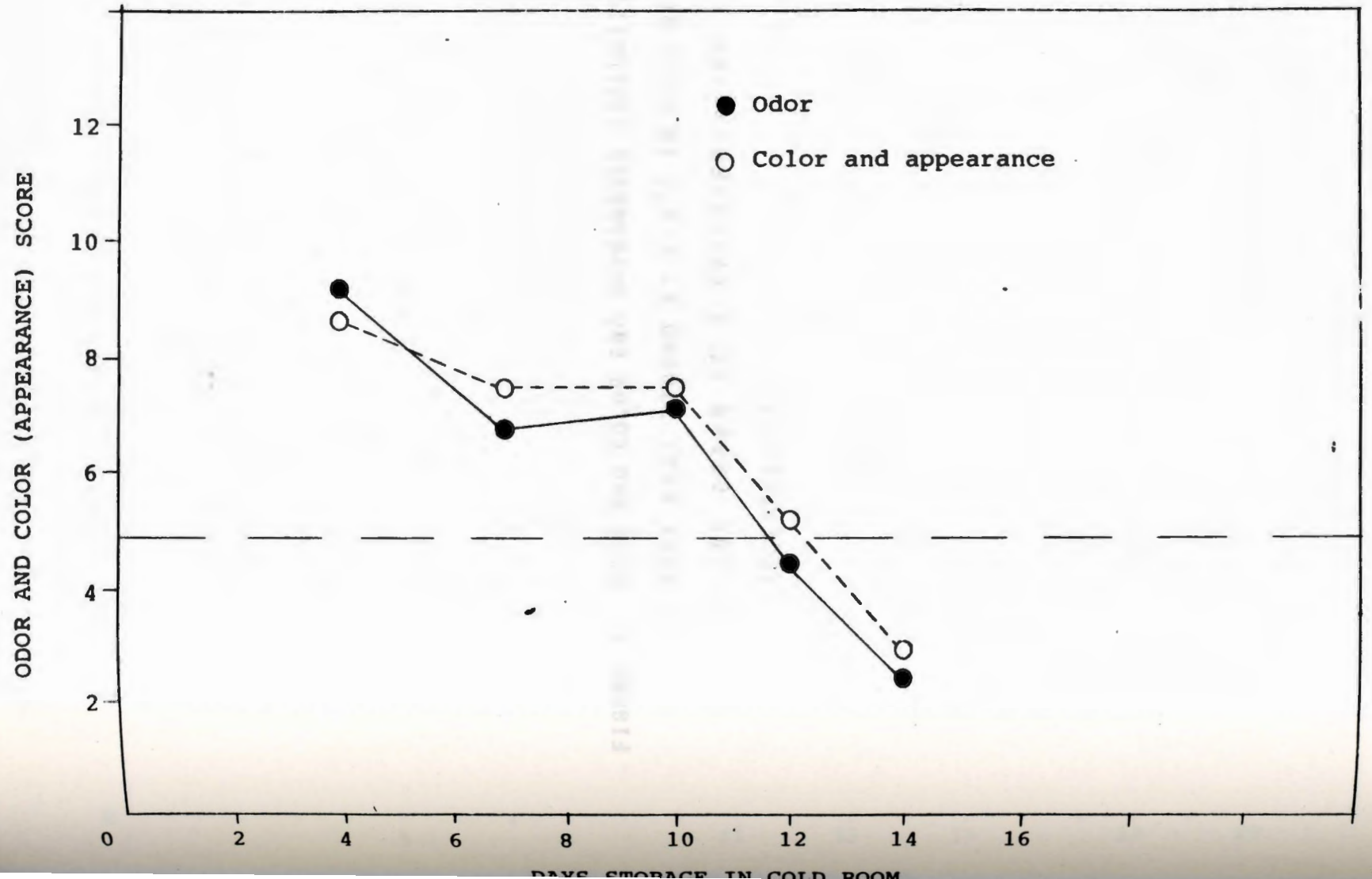


FIGURE 2. ODOR AND COLOR AND APPEARANCE EVALUATION OF RAW LAMB MEAT SOTRED AT 2-4°C. THE LINE DRAWN AT 5 REPRESENTS THE LIMIT OF ACCEPTABILITY.


Odor
Color and appearance
ODOR AND COLOR (APPEARANCE) SCORE
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
DAYS STORAGE IN COLD ROOM

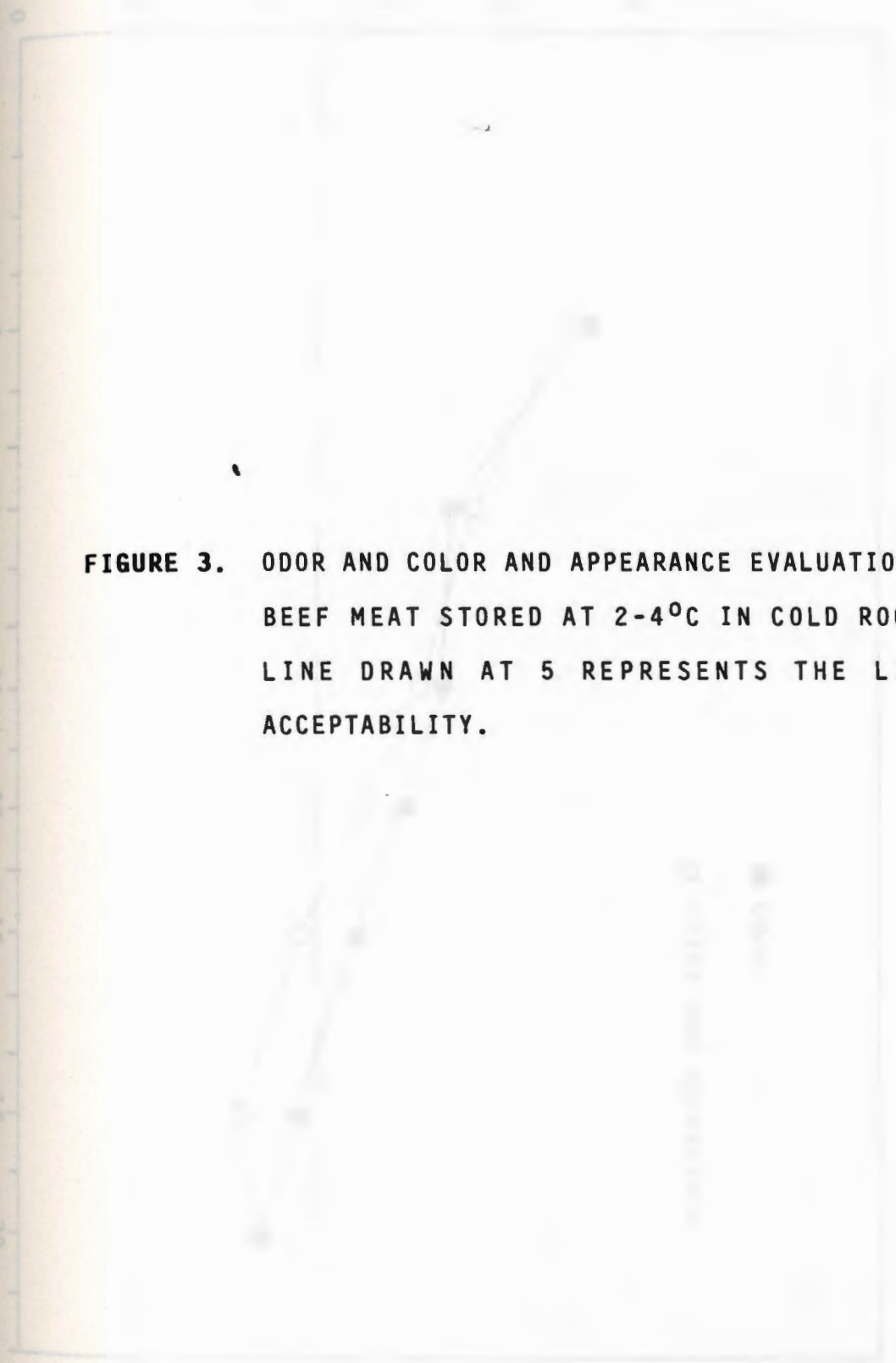
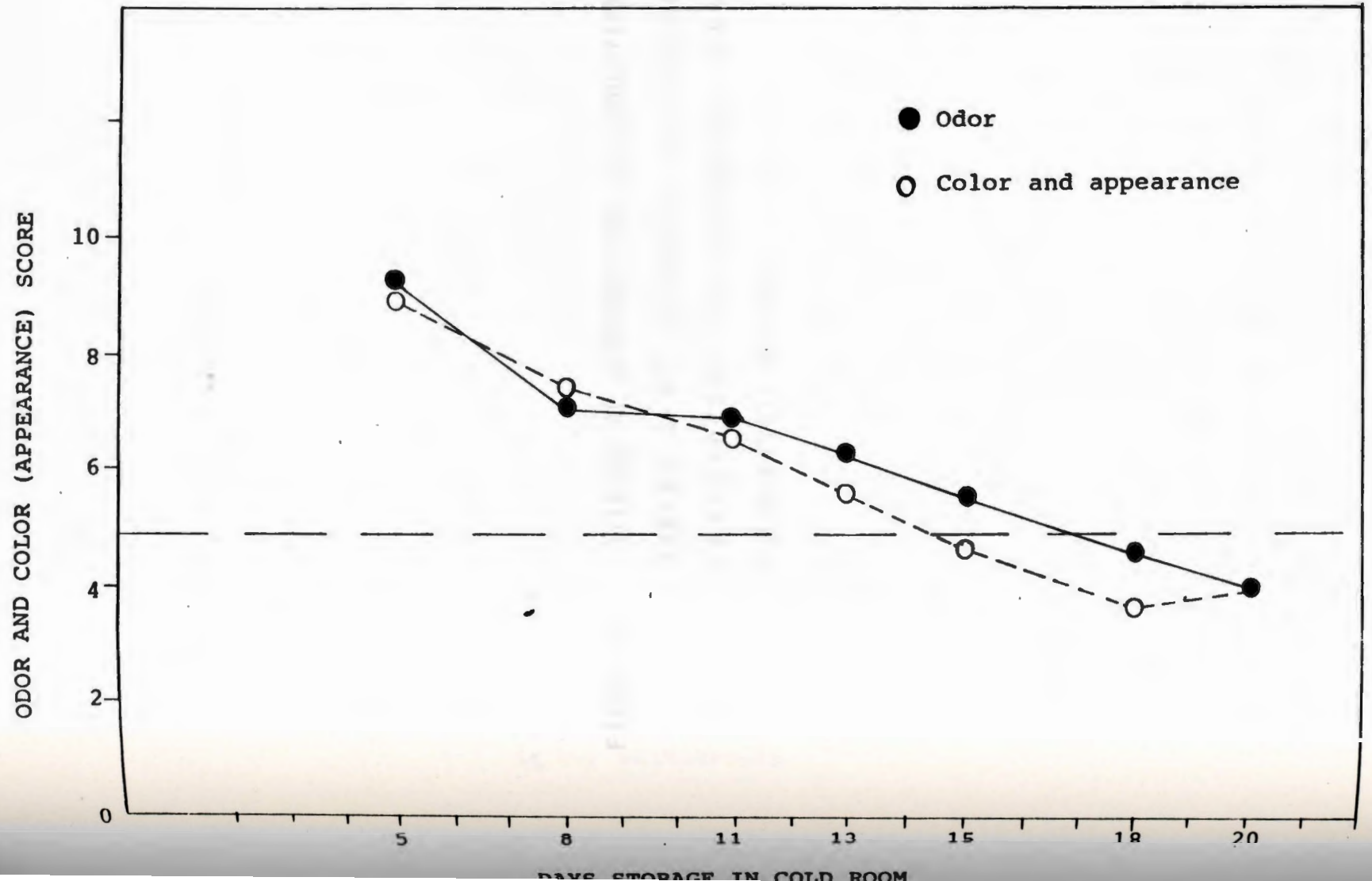


FIGURE 3. ODOR AND COLOR AND APPEARANCE EVALUATION OF RAW BEEF MEAT STORED AT 2-4°C IN COLD ROOM. THE LINE DRAWN AT 5 REPRESENTS THE LIMIT OF ACCEPTABILITY.


Odor
Color and appearance
ODOR AND COLOR (APPEARANCE) SCORE
0
2
4
6
8
10
5
8
11
13
15
18
20
DAYS STORAGE IN COLD ROOM

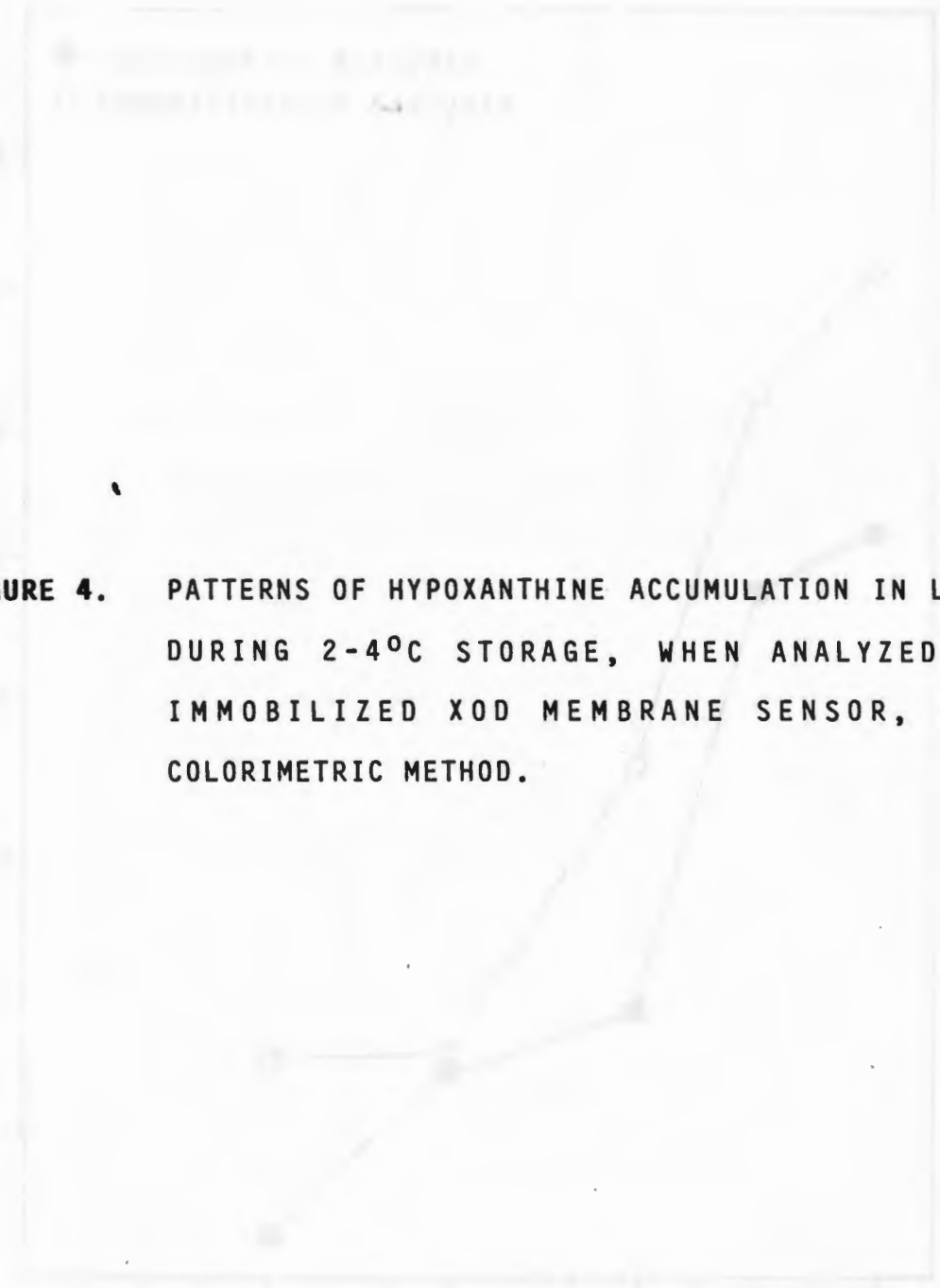


FIGURE 4. PATTERNS OF HYPOXANTHINE ACCUMULATION IN LAMB DURING 2-4°C STORAGE, WHEN ANALYZED BY IMMOBILIZED XOD MEMBRANE SENSOR, AND COLORIMETRIC METHOD.

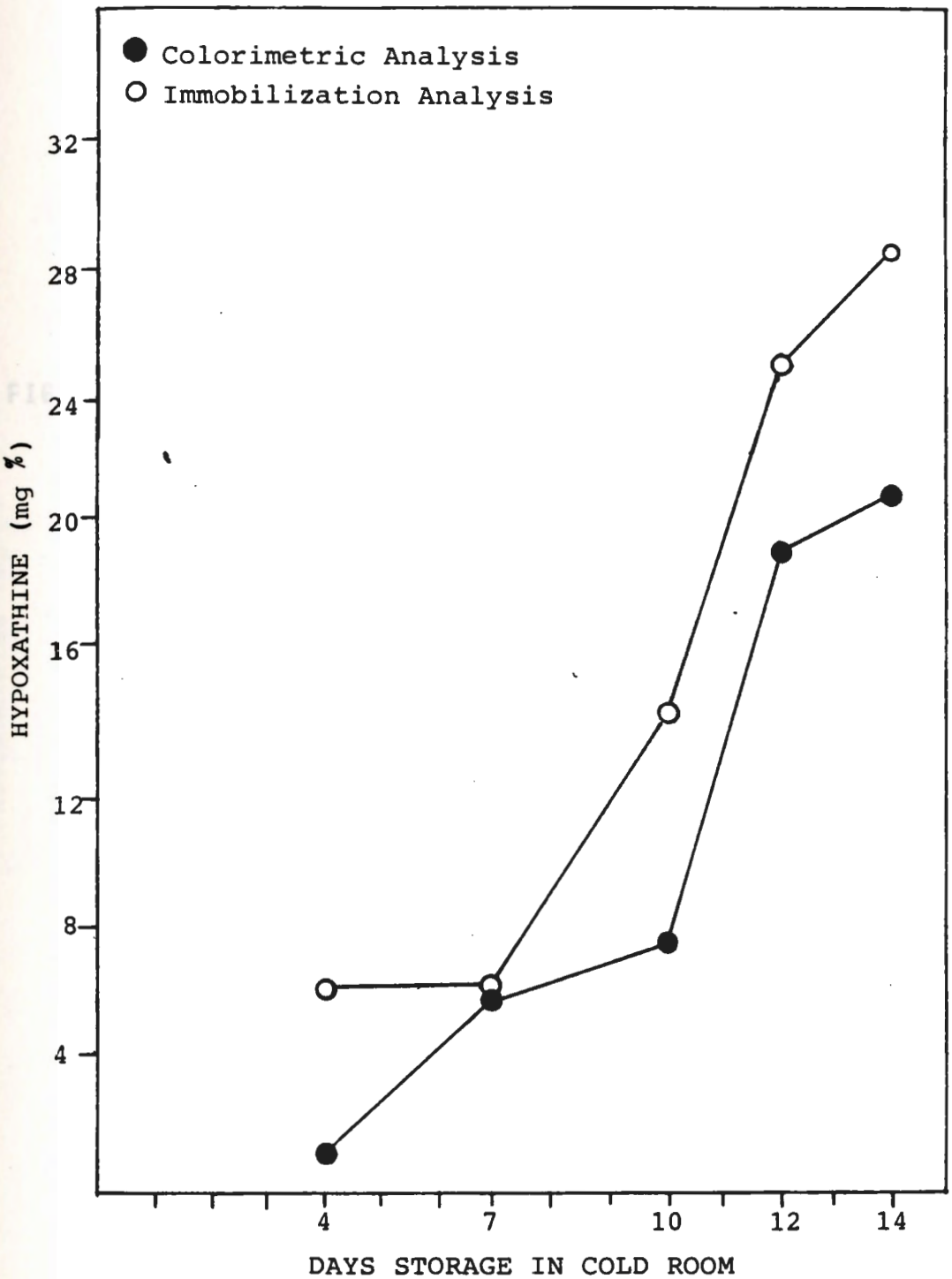
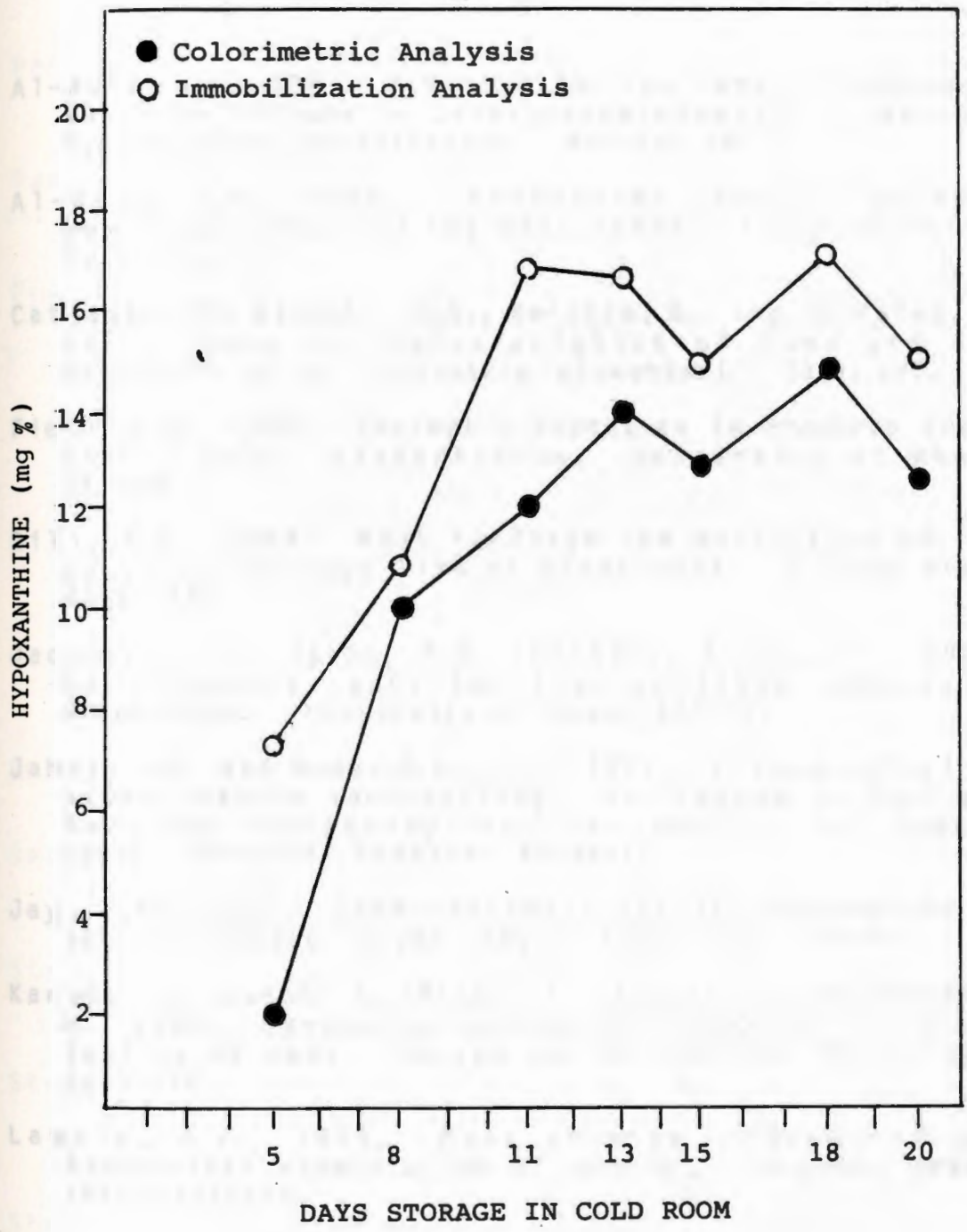

Colorimetric Analysis
Immobilization Analysis
32
28
24
20
16
12
8
4
HYPOXATHINE (mg %)
4
7
10
12
14
DAYS STORAGE IN COLD ROOM

FIGURE 5. PATTERNS OF HYPOXANTHINE ACCUMULATION IN BEEF DURING 2-4°C STORAGE WHEN ANALYZED BY IMMOBILIZED XOD MEMBRANE SENSOR, AND COLORIMETRIC METHOD.


● Colorimetric Analysis
○ Immobilization Analysis
HYPOXANTHINE (mg %)
20
18
16
14
12
10
8
6
4
2
5
8
11
13
15
18
20
DAYS STORAGE IN COLD ROOM

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APPENDIX I

Literature Review

Literature Reviewed

Enzymes have been useful as catalysts in many reactions because they possess high degrees of specificity in the reaction, and high catalytic activity and work under mind conditions of termperature, pH and pressure. However, enzymes have not been used because they are water soluble molecules that are difficult to recover and use again. Also, most of them are not stable under operational conditions. However, the main reason for not using them in all operations is their cost.

Therefore, it would be a great advantage if we could recover the expensive enzyme after it has performed its task and reuse it. In this way the amount of material processed per unit mass of enzyme would be greatly increased. Efforts to realize this aim have led to the development of immobilized enzyme technology. The concept is a simple one. The enzyme is first attached to, or trapped within, an insoluble carrier so that it can be removed from the mixture when the enzyme treatment has progressed to the required extent. An additional advantage of such a system is that it opens the way to continuous processing whereby the material to be treated is passed through a reactor containing the immobilized enzyme. The extent of the enzymatic reaction is then

controlled by the rate of flow through the reactor since this determines the time the enzyme is in contact with its substrate. The lack of mobility of the bound enzyme is offset by a high ratio of enzyme to substrate in the reactor (Beeby, 1983).

The first two papers on immobilized enzymes were published by Nelson and Griffin (1916), and Nelson and Hitchcock (1921). Both described the absorption of invertase on charcoal and aluminum hydroxyl gel. Campbell et al. (1951) tried to isolate antibodies by covalently binding an antigen to celulose. Grubhofer and Schleith (1953) from Germany immobilized enzymes such as pepsini, diastase, carboxypeptidase and ribonuclease covalently to an insoluble matrix, but the actual breakthrough in enzyme technology developed about twenty-five years ago, was known as enzyme immobilization.

Since the early 1960s, Chibata and his colleague have investigated immobilized enzymes with the aim of utilizing them for continuous industrial production. The first report on immobilized aminoacylase was presented at the annual meeting of the Agricultural Chemical Society of Japan in 1965 and published in Enzymologia in 1966. In 1969 they succeeded in the industrialization of a continuous process for the optical resolution of DL-amino acid using immobilized aminoacylase. This was the first

industrial application of immobilized enzymes in the world. In the late 1960s, work on immobilized enzymes was carried out extensively in the U.S.A. and Europe, and the number of reports on immobilized enzymes increased markedly. Beside these reports, many reviews and books have been published. In 1971 at the first Enzyme Engineering Conference, held at Henniker, New Hampshire, U.S.A., the predominant theme was immobilized enzymes and the definition and classification of immobilized enzymes were proposed (Chibata, 1978).

Whereas enzymes convert from water soluble to water insoluble molecules, several definitions are necessary for an immobilized enzyme. An immobilized enzyme is an enzyme that has been chemically or physically attached to a water-insoluble gel matrix or water insoluble microcapsule (Zaborsky, 1973). Enzyme immobilization is the imprisonment of an enzyme molecule in a distinct phase that allows exchange with, but is separated from, the bulk phase in which a substrate effector or inhibitor molecules are dispersed and monitored (Trevan, 1980). Immobilization is the conversion of enzymes from a water-soluble, mobile state to a water-insoluble, immobile state (Klibanov, 1983).

To immobilize the enzymes, many different immobilization techniques and procedures have been used

and discussed (Carr and Powers, 1980; Hultin, 1974; Klibanov, 1983; Laskin et al., 1984; Mosbach, 1976; Trevan, 1980; Wingard, 1972; Zaborsky, 1973.

These techniques and procedures which are used for the immobilization have been mainly classified and grouped as seen in the following tables and figures:

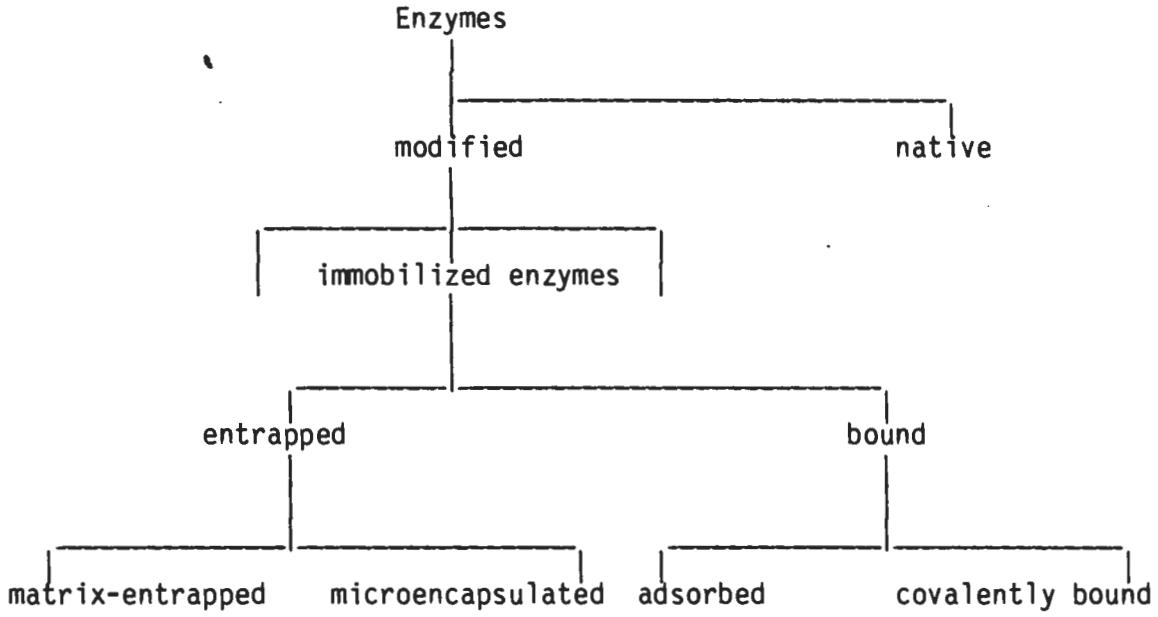


Fig. 1 Classification of immobilized enzymes from Enzyme Engineering. Edited by L.B. Wingard, J. Wiley & Sons, 1972, Fig. 1.

Table 1

CLASSIFICATION SYSTEMS OF THE METHODS EMPLOYED FOR THE IMMOBILIZATION OF ENZYMES

Chemical methods (covalent bond formation-dependent)

Attachment of enzyme to water-insoluble, functionalized polymer.

Incorporation of enzyme into growing polymer chain.

Intermolecular crosslinking of enzyme with a multifunctional, low molecular weight reagent.

Physical methods - (noncovalent bond formation-dependent).

ADsorption of enzyme onto water insoluble matrix.

Entrapment of enzyme within water-insoluble gel matrix (lattice-entrapment).

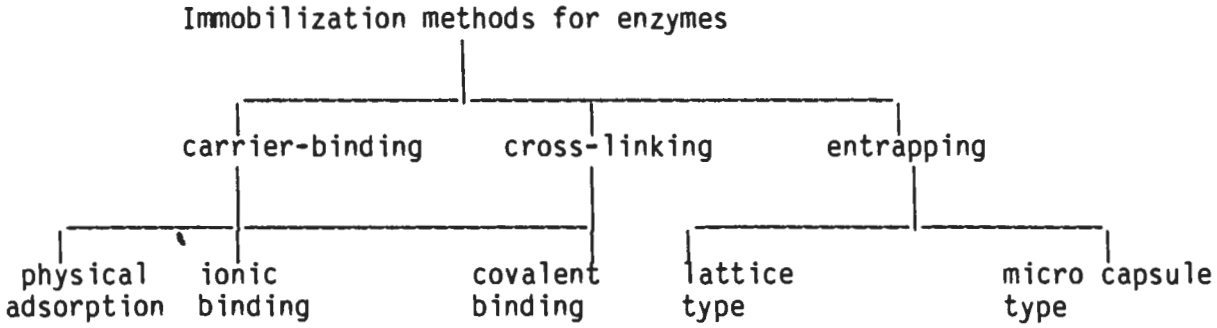
Entrapment of enzyme within permanent and nonpermanent semipermeable microcapsules.

Containment of enzyme within special semipermeable membrane dependent devices.

From Immobilized Enzymes by O.R. Zaborsky, CRC Press, Cleveland, Ohio, 1973, Table 1.)

Figure 2

CLASSIFICATION OF IMMOBILIZATION ENZYMES



From Immobilized Enzymes, Edited by I. Chibata, J. Wiley & Sons, 1978, Fig. 1.3.

Table 2

CLASSIFICATION OF IMMOBILIZATION ENZYMES

1 - Covalent attachment of enzymes to solid supports.

2 - Adsorption of enzymes on solid supports.

3 - Entrapment of enzymes in polymeric gel

4 - Cross-linking of enzymes with bifunctional reagents.

5 - Encapsulation of enzymes.

From Immobilized Enzymes and Cells as Practical Catalysts, by A.M. Klibanov, in Science, vol. 219, pp. 722-727, 1983.

Each one of these methods has its advantages and no one is best for all applications. Klibanov (1983) reported that comparison of the enzymes immobilization methods listed in Table 2 leads to some important conclusions. The advantage of covalent methods 1 and 4 is that they result in stronger chemical bonds between the enzyme and the support. The disadvantages are that covalent binding is relatively laborious and expensive and often leads to significant inactivation of enzymes due to attachment through their active centers. The latter problem, however, can be alleviated in many cases if immobilization is carried out in the presence of substrates or other ligands (inhibitors, cofactors, and so on) that selectively protect the active center from the attachment. Methods of immobilization such as adsorption and gel entrapment are very simple and efficient, but since such methods create no strong bonds between the enzyme and the matrix, enzymes often leak from the supports. This problem can be overcome by the treatment of adsorbed or entrapped enzymes with a cross-linking reagent such as glutaraldehyde.

Preparation methods and characteristics of immobilized enzymes are summarized broadly in Table 3, though there are many exceptions. Although a number of enzyme immobilization methods have been studied, no ideal

general methods applicable for the immobilization of many enzymes have yet been developed. Each method has specific disadvantages. Therefore, in practice, it is necessary to find a suitable method and conditions for the immobilization of a particular enzyme in light of the intended application (Chibata, 1978).

Table 3

PREPARATION AND CHARACTERISTICS OF IMMOBILIZED ENZYNES

Characteristic	Carrier binding method			Cross linking Method	Entrapping Method
	Physical adsorption	Ionic binding	Covalent binding		
Preparation	easy	easy	difficult	difficult	difficult
Enzyme activity	low	high	high	moderate	high
Substrate specificity	unchangeable	unchangeable	changeable	changeable	unchangeable
Binding force	weak	moderate	strong	strong	strong
Regeneration	possible	possible	impossible	impossible	impossible
General applicability	low	moderate	moderate	low	high
Cost of immobilization	low	low	high	moderate	low

After studying all the methods of immobilization presented thus far, the entrapment method was chosen.

To entrap an enzyme in polymeric gel, the enzyme is added to a solution of monomers before the gel is formed. Then gel formation is initiated by either changing the temperature or adding a gel-inducing chemical. As a result, the enzyme becomes trapped in the gel volume. The gels employed for immobilization of enzymes may be covalent (for instance, polyacrylamide cross-linked with N, N^1-methylenebisacrylamide) or non-covalent (calcium alginate or kappa-carrageenan), (Klibanov, 1983).

The matrices which have been most employed in entrapment methods are polyacrylamide, collagen, cellulose triacetate, agar and alginate. The first polymeric matrix used to immobilize enzyme was polyacrylamide (Bernfield and Wan, 1973). Polyacrylamide, Kappa-carrageenan and alginate have been used industrially (Klibanov, 1983).

Recently more attention has been focused on alginate gel (ca-alginate) especially in producing ethanol (Hahn-Hagerdal, 1984; Kierstan and Bucke, 1977; Klein and Kressdorf, 1983; McGhee et al., 1984; McGhee et al., 1982; Williams and Munnecke, 1981; White and Portno, 1978) and citric acid (Vaija et al., 1982; Eikmeier and Rehm, 1984), and in a milk assay (Jacober & Rand, 1984).

Alginate immobilization was chosen because of its simplicity, because the immobilization reagent are of low cost, making the procedure attractive for large scale application, and because the alginate gel immobilization procedure is mild (Kiersta, 1981).

Brodelius (1984) summarized and discussed some factors that influence the choice of immobilization methods for a large-scale operation. These are:

1. Ease of preparation,
2. Toxicity,
3. Cost of polymer, and
4. Mechanical strength of gel.

A gel meeting these criteria is ca-alginate. The preparation of an alginate-entrapped cell is very easy and can be employed on a large scale without complication. The alginate-entrapped cells are easily prepared in the volume reactor. The price of alginate is low, and it is available in unlimited amounts. The mechanical stability of alginate is acceptable and, furthermore, it is widely used as a food additive. A product from a process based on alginate-entrapped cells may therefore pass the FDA more easily.

A few years ago, the use of biochemical electrodes having bio-specificity attracted considerable attention for checking or controling the concentration of

metabolites in body fluids. This bio-specificity is based on enzymes, and immobilized enzymes are used in many cases. These electrodes are suitable for the measurement of substrates, coenzymes, and the inhibitors of an enzyme, and are called "enzyme electrodes" or "microbial electrodes" when microbial cells are used (Chibata, 1978). Clark and Lyons (1962) proposed the first amperometric enzyme electrode. Since then the literature reviewed shows numerous references to enzyme electrode production and use (Olson and Richardson, 1974; Taylor, et al. 1977; Hasselberger, 1978; Car and Bowrers, 1980; Wingard et al., 1981; Laskin et al., 1984).

Since the YSI-Clark 2510 electrode and YSI Model 25 oxidase meter (Yellow Springs, Ohio), which were developed by this company, were used for this study, the principles and operation of the system as described by this company are summarized as follows:

The system consists of the YSI-Clark 2510 oxidase probe and the YSI Model 25 oxidase meter for amplification and display of the probe signal. The probe is a 2-electrode linear amperometric (polarographic) device with an enzyme retraining system based on a design by Dr. Leland C. Clark, Jr., Children's Hospital Research Foundation, Cincinnati, Ohio (U.S. Patent).

The YSI-Clark 2510 oxidase probe consists of a

platinum anode of 0.06" dia. surrounded by a silver cathode approximately 0.275" dia. The end of the probe is covered by a membrane secured with an "o" ring as shown in Fig 4. When a polarizing voltage is applied the probe oxidizes a constant portion of the H_2O_2 at the platinum anode, Reaction I. The resulting current is directly proportional to the H_2O_2 present.

Reaction I $\quad H_2O_2 \longrightarrow O_2 + 2H^{=} + 2e$

The circuit is completed by a silver cathode at which oxygen is reduced to water, reaction II.

Reaction II $\quad 2e^{-} + 2H^{+} + 1/2\ O_2 \longrightarrow H_2O$

The membrane covering the probe is porous, serving both to protect the electrodes and to define a diffusion path to them. Various methods can be used to introduce the enzyme(s) necessary to the system. They can be placed behind the membrane, in the bulk solution being measured, or both.

The level of the final, stable current reading and the time required to attain it depends upon whether the enzyme is placed in the solution or isolated behind the membrane. In the bulk solution the enzyme is relatively

less concentrated and a longer time is required to attain a steady state. Also the final current is higher because the conversion is essentially total. Behind the membrane the enzyme is highly concentrated and converts only the substrate which has diffused through the membrane. In this case the steady state is reached more quickly but the final current is lower.

The system is a research tool (not for diagnostic use) specifically designed to study substances that produce or consume, or influence production or consumption of H_2O_2. In operation the probe produces an electric current proportional to the H_2O_2 in its immediate vicinity. When an enzyme is used to produce H_2O_2, the probe current is a measure either of enzyme activity or substrate concentration, depending upon the experiment design, reaction III.

Reaction III Oxidase substrate + O_2 $\xrightarrow[\text{enzyme}]{\text{oxidase}}$ product + H_2O_2

Determination of enzyme activity also gives indirect information about the presence and function of enzyme cofactors and inhibitors.

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