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FINAL RESEARCH PROGRESS REPORT ON THE REVERSIBLE INACTIVATION ON IRON ENZYME WITH AMINO ACID ANTAGONIST BY MEANS OF ELECTROLYTIC PROCESS,- CONTRACT Nonr-(00) - PROJECT NUMBER NR-122-075, BY SABURO KATSURA, RESEARCH ASSOCIATE, AND HAROLD F. WALTON, PROJECT SUPERVISOR, DEPARTMENT OF CHEMISTRY, UNIVERSITY OF COLORADO, MARCH 15, 1953.

The following report covers the period from September 1, 1952, to December 31, 1952.

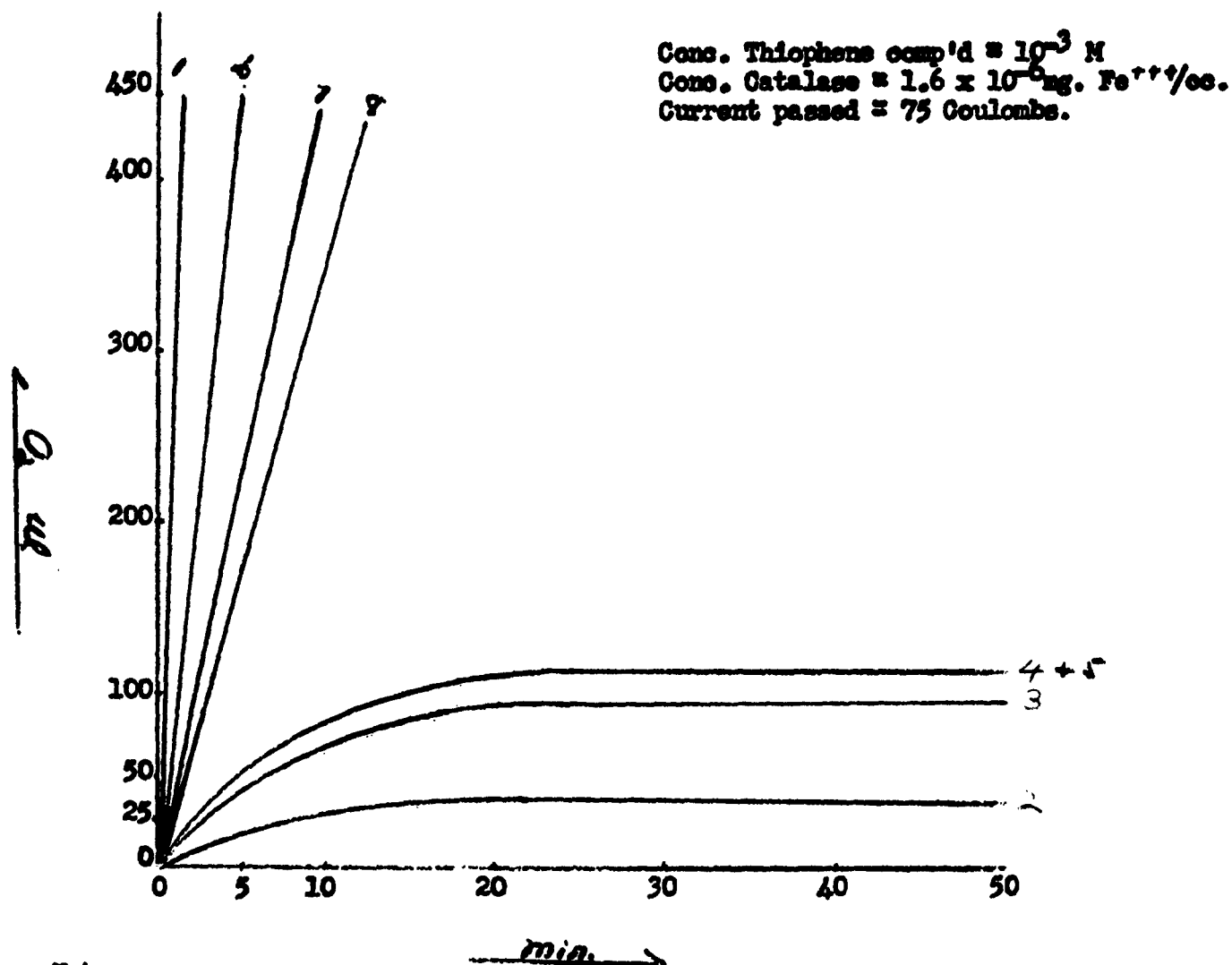
CONFIRMATORY STUDIES ON THE ANODIC INACTIVATION OF CATALASE WITH THIOPHENE COMPOUNDS.

The hypothesis maintained in this laboratory that the inactivated form of catalase, through the process of 4-electrode cell in presence of thienylalanine, must have the prosthetic iron in reduced form has been further confirmed. Ferric chloride, substituted for catalase under the indentical procedure of electrolysis is reduced to ferrous; the evidence of this is demonstrated by adding ortho-phenanthroline, which gives a characteristic red color with ferrous ions but not with ferric. The substitution for thienylalanine is also tried with analogous compounds, such as thiophene, acetylthiophene, thiophenecarboxylic acid, and found that each of these compounds is almost equally effective in inactivating catalase, (Graph 1.)

REACTIVATION WITH COPPER ANODE AND COPPER SULFATE

Catalase inactivated through electrolysis in presence of a thiophene compound is readily reactivated when the solution containing the inactivated catalase is subjected again to electrolysis with the copper anode. The reactivation is also possible when the solution with inactivated catalase is treated with copper sulfate, (Graph 1.) The results of the use of nickel sulfate as well as silver sulfate to restore the lost activity of catalase are ambiguous. These studies require further investigation.

Graph 1. Manometric Measurement of Decomposition Velocity H_2O_2 with Fresh Catalase and Inactivated and Reactivated Catalase.



Note:

- 1 - Control (Fresh catalase.)
- 2 - Catalase inactivated with thienylalanine.
- 3 - Catalase inactivated with thiophene.
- 4 - Catalase inactivated with acetylthiophene.
- 5 - Catalase inactivated with thiophene carboxylic acid.
- 6 - Reactivation of 2 at copper anode.
- 7 - Reactivation of 3, 4, and 5, at copper anode.
- 8 - Reactivation of 2, 3, 4, and 5, with copper sulfate.

pH CHANGE OCCURRING IN PERIODIC CYCLE CONTROLLED THROUGH INTERRUPTED CURRENT

How the constant change in H ion concentration in the conventional 2-electrode cell as the result of passing current through phosphate buffer caused a drastic change in pH value of the solution has been already reported.

Graph 2 shows how the change in pH within controlled range takes place both in anode and cathode in 4-electrode cell. The curve in this graph is measured 30 minutes after the current is passed. The curve however remains the same even after the 24th hour in continued electrolysis.

In this study, phosphate buffer, 5×10^{-5} M, is used with the pH value of the solution adjusted to 7 before the current, 5 ma, is passed. In 4-electrode cell the current flows interchangeably (see the detail description in report, September 25, 1951.)

The automatic re-adjustment of the pH value both in anode and cathode by the current takes about 30 minutes after the current is commenced to flow. After this re-adjustment, the range of pH change is automatically controlled. In this case, the extent of pH change is 6.7 to 6.75 at anode, and 7.25 to 7.30 at cathode.

The maximum pH point, 6.75, at anode is reached at the 60th second, i. e. the end of the interrupted period during of which the positive electrode is out of circuit. After this period is over the positive electrode goes in circuit for the next 60 seconds to change the pH value from 6.75 to 6.70, the minimum value, which is reached at the end of the 60th second just before the current interruption.

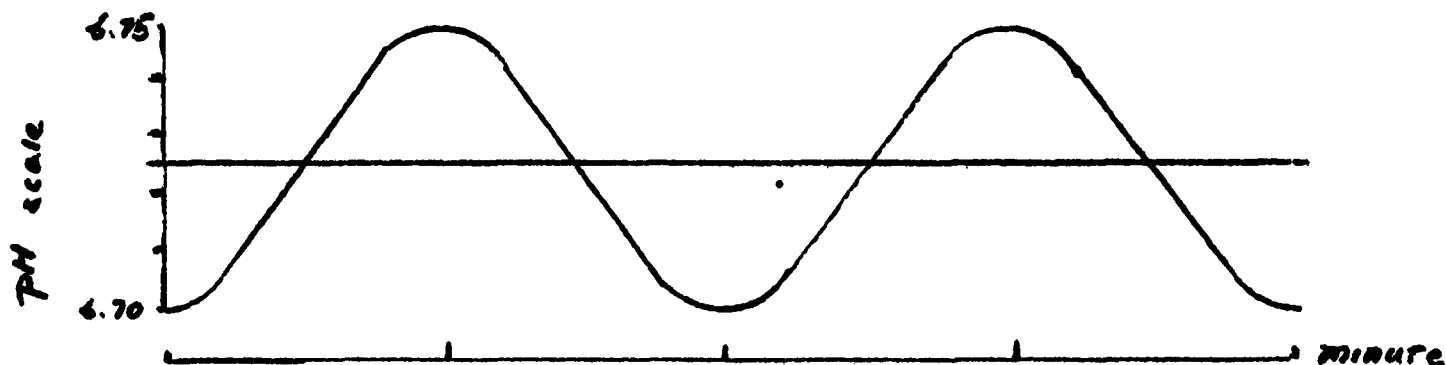
Exactly the reverse reaction takes place at cathode, which is separated with the permeable cellophane casing in which the negative electrode is at rest while the opposite electrode in the next chamber is in circuit. The pH value in this chamber is slightly basic and the controlled range of change is 7.25 to 7.30. The maximum point in the graph is reached at the end of the 60th second when the negative electrode is in circuit. The change to the minimum occurs at the end of the interrupted 60 seconds.

In either case, anode or cathode, the periodic cycle is observed in symmetrical sine wave with the stabilized x-axis, which at anode is slightly acidic - the optimum pH for catalase. From this curve it is obvious that the point of inflection in both concave-upward and downward must occur at the 30th second in each minute.

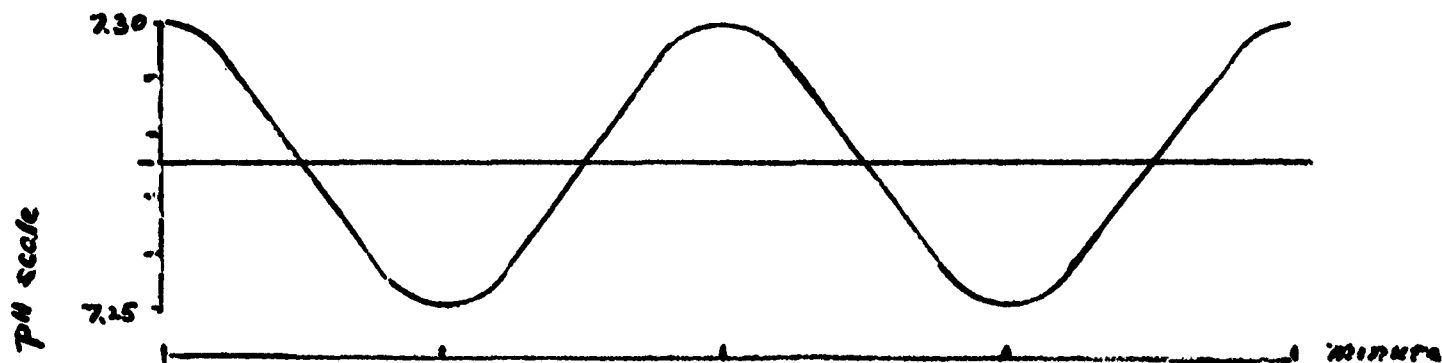
Graph 2. pH CHANGE IN 4-ELECTRODE CELL SHOWN IN PERIODIC CYCLE.

Phosphate buffer = 5×10^{-5} M
pH 7 (before current is passed)
Current, interrupted = 5 ma.

Anode



Cathode



STUDIES ON ELECTROLYZED THIOPHENE COMPOUNDS THROUGH U-V ABSORPTION SPECTRA,
POLAROGRAPHIC ANALYSIS, AND MANOMETRIC MEASUREMENT OF CATALYTIC ACTIVITY OF
CATALASE

2-electrode cell:

The absorption bands of the fresh and electrolyzed thienylalanine is shown in graph 3. The fresh thienylalanine, not electrolyzed, shows its peak at $235\text{m}\mu$. The peak is decidedly displaced after thienylalanine is electrolyzed. It is difficult to tell just where the peak is situated in electrolyzed thienylalanine. In this graph the peak is at $215\text{m}\mu$, but the true peak may be found beyond this range. However it is believed that this curve with displaced peak is the oxidized form of thienylalanine. The electrolyzed thienylalanine is reducible at the dropping mercury cathode (graph 4,) but the fresh thienylalanine is not reducible.

The absorption bands of the fresh and electrolyzed thiophene, appearing very much the same as those bands of thienylalanine, are shown in graph 5. Like thienylalanine, thiophene does not reduce at the dropping mercury cathode, but the electrolyzed thiophene from anode does reduce at the dropping mercury cathode, see graph 4.

4-electrode cell:

The absorption band of thienylalanine from anode where it is subjected to electrolysis in presence of catalase still retains the peak at $235\text{m}\mu$, although the curve is greatly suppressed. Attempts have been made to obtain the curve with displaced peak as in the case with 2-electrode cell by passing the current as much as 75 coulombs. But the suppressed curve still retains the original peak, (graph 6.)

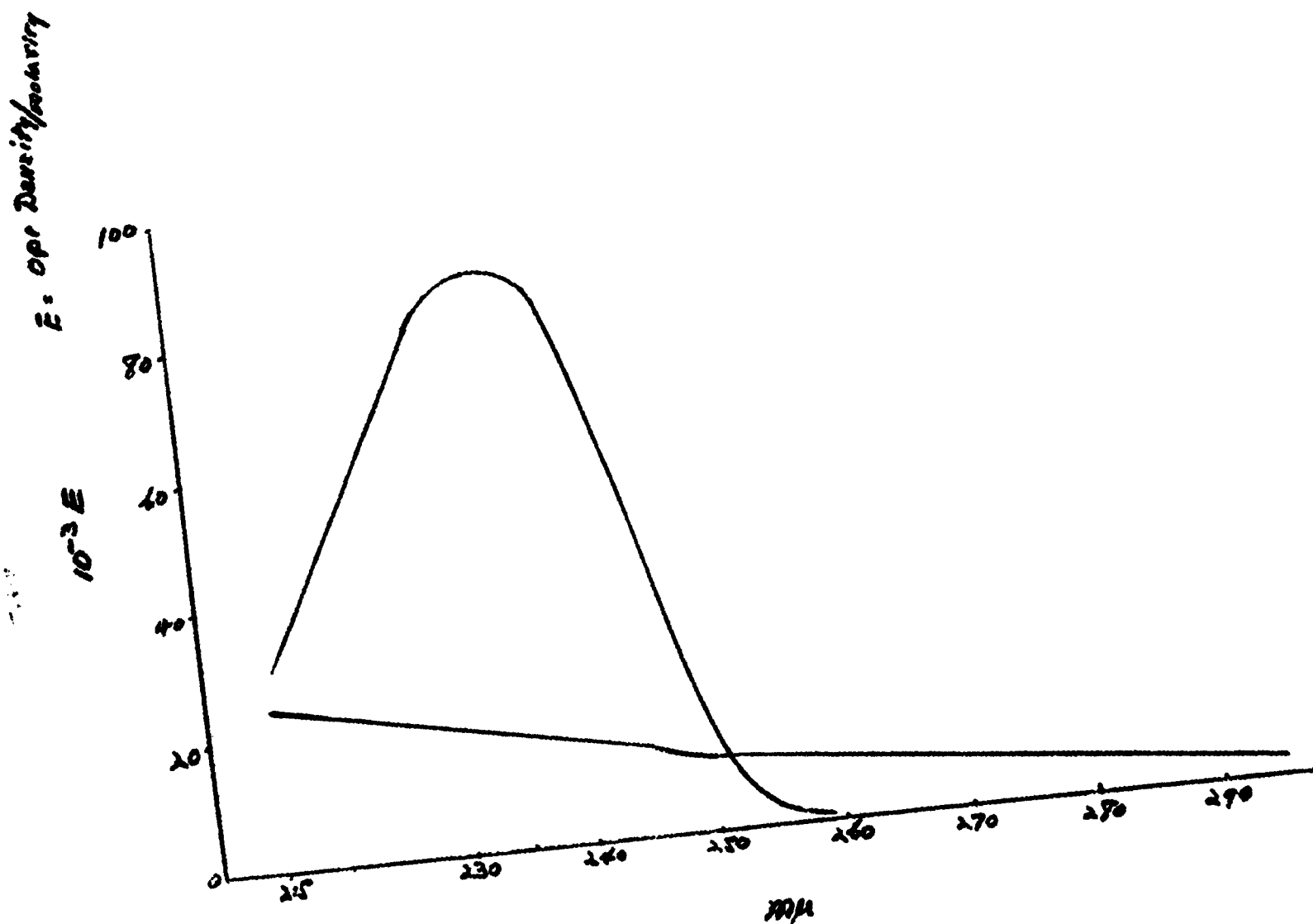
The absorption in this case must have been disturbed with the presence of catalase. At this wavelength region catalase with the concentration as low as used in this experiment is not visible. Therefore, this suppressed curve must be of a catalase-antagonist complex.

A polarographic analysis of the thienylalanine electrolyzed in presence of catalase shows the reduction wave equally prominent as the thienylalanine electrolyzed without catalase, (graph 7.) The catalytic activity from this solution is inhibited as shown in graph 1.

Graph 3. U-V Absorption of Thienylalanine.

The curve with its peak at $235 m\mu$ is:
fresh thienylalanine, 10^{-5} M.

The suppressed curve is:
thienylalanine, 10^{-5} M, after
electrolysis.
From anode (current: 25 coulombs.)



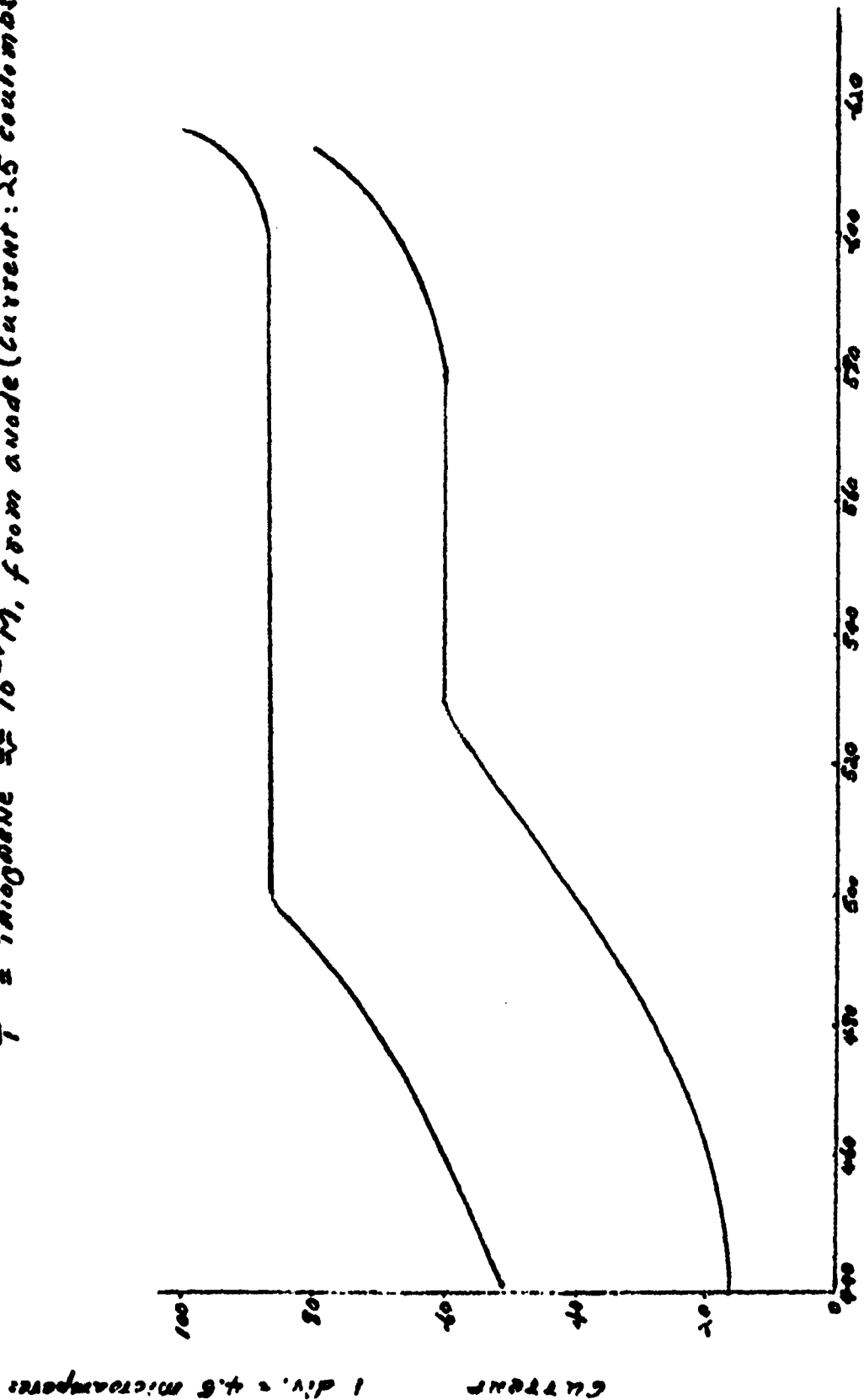
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Graph 4

Polarographic analysis of thiophene compds after electrolysis

TA = Thiengalanine, $10^{-6} M$, from anode (current: 25 coulombs)

T = Thiogbene $\approx 10^{-7} M$, from anode (current: 25 coulombs)

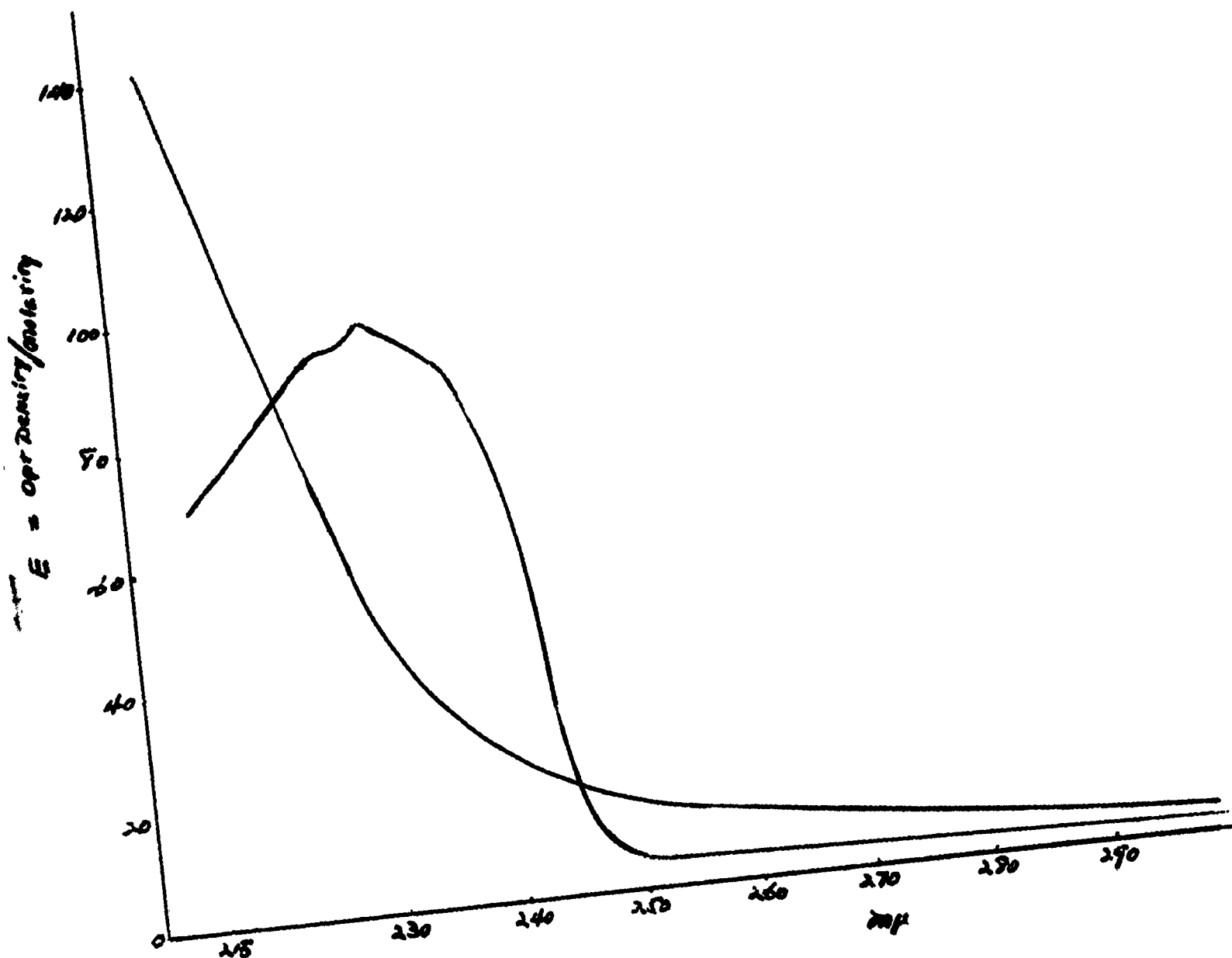


$E = f(\text{span EMF})$
span EMF = 3 v

Graph 5. U-V absorption of Thiophene.

The curve with its peak at $230 m\mu$ is:
fresh thiophene, $\approx 10^{-5} M$.

The suppressed curve is:
thiophene $\approx 10^{-5} M$ after
electrolysis.
From anode (current: 25 coulombs.)

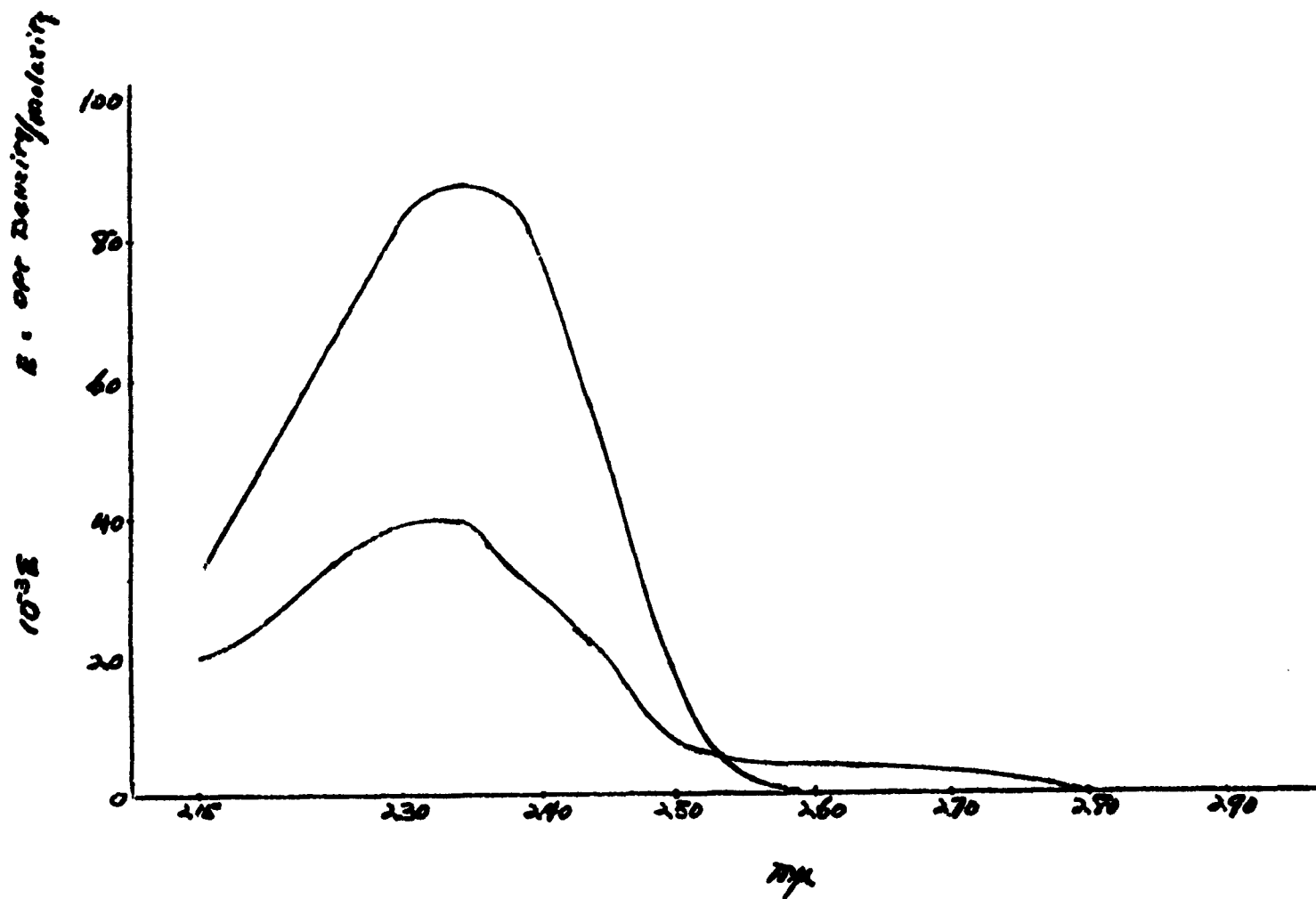


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Graph 6. U-V absorption of Thienylalanine electrolyzed with catalase.

The curve with its peak at 235 $m\mu$ is:
fresh thienylalanine, 10^{-5} M.

The suppressed curve is:
thienylalanine, 10^{-5} M, electrolyzed
in presence of catalase.
From anode (current: 75 coulombs.)
Conc. catalase $\approx 1.6 \times 10^{-8}$ mg Fe⁺⁺⁺/cc.

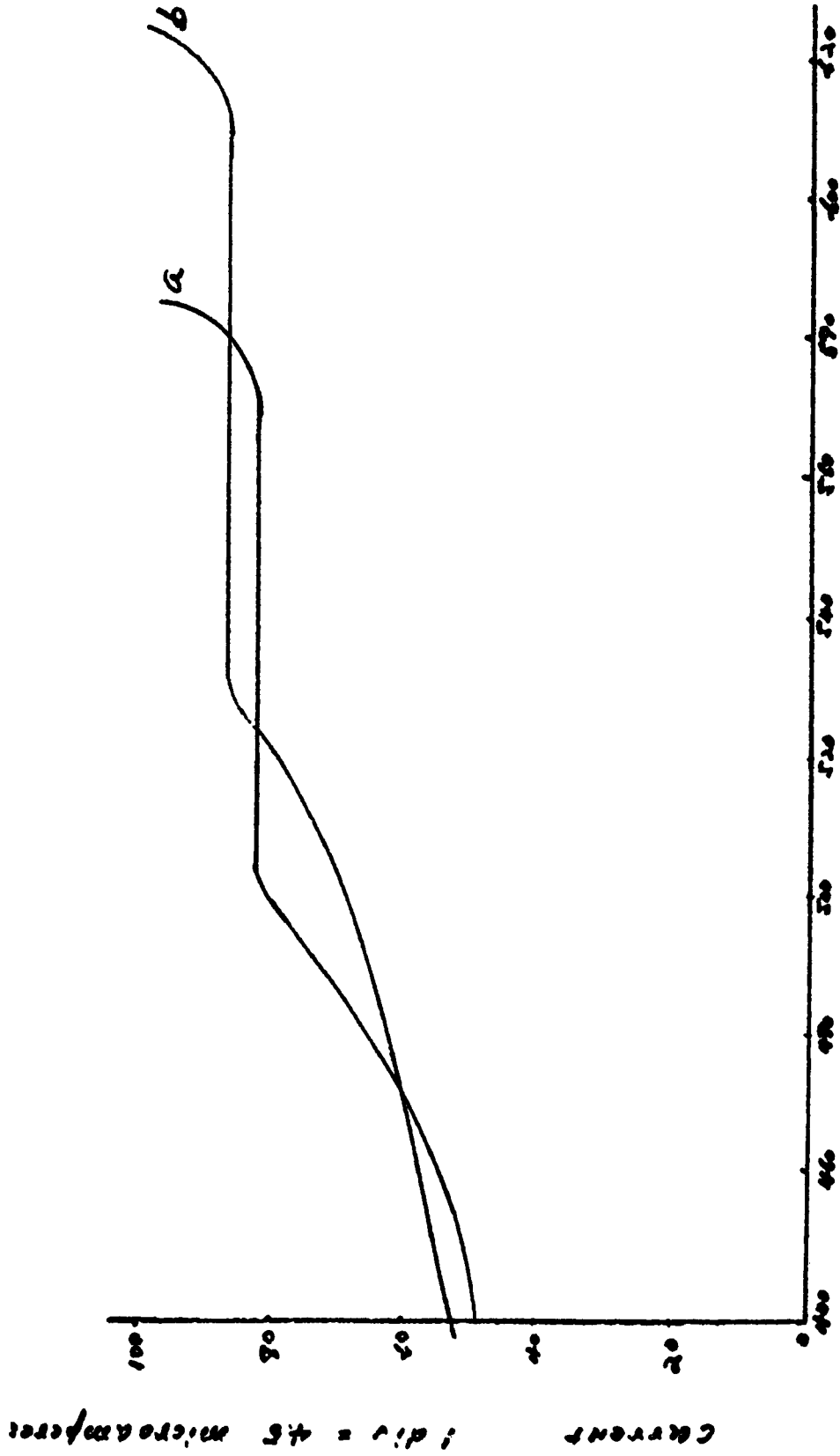


Graph 7.

Polarographic analysis of thiouygalanine electrolyzed with and without catalase.

a = Thiouygalanine, $10^{-6}M$ + Catalase, $(1.8 \times 10^{-9} \text{ mg } Fe^{+++}/cc.)$ from anode (current: 75 coulombs)

b = Thiouygalanine, $10^{-6}M$ (without catalase) from anode (current: 75 coulombs)



$E = f(\text{open EMF})$

Open EMF = 3 V

TENTATIVE CONCLUSION ON THE ANODIC INACTIVATION OF CATALASE RESULTING IN REDUCTION OF THE PROSTHETIC IRON AS THE RESULT OF THE OXIDATION OF THIENYLALANINE DURING THE ELECTROLYSIS

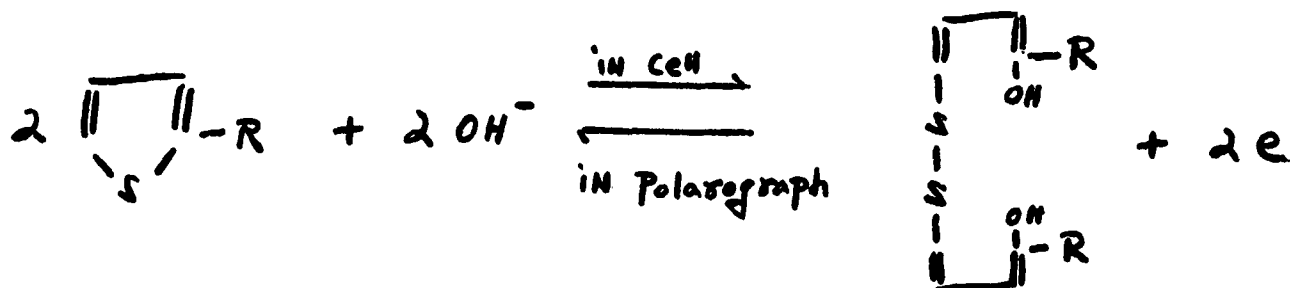
The reaction kinetics of enzyme systems in which enzyme-substrate compounds may be involved have been widely studied but the results have been always doubtful; in some cases the enzyme-substrate compounds act catalytically and in other cases the enzyme substrate can act as inhibitors of enzymatic function.

In our experiment what we speculate as catalase-antagonist complex, mentioned in page 5 in this report, does not come under the category of the enzyme-substrate complex. The amino acid antagonist used in this experiment cannot be called a substrate to catalase because the reduction of catalase iron, which immediately results in its inactivation of catalytic activity, occurs only when electrical energy is used. In this case it is very likely a complex, if it actually occurs, is formed with the product of electrolysis from catalase proper and this formation of complex is not responsible for the inhibition of catalytic action of catalase; the occurrence of a similar complex has been previously observed when allylglycine or furylalanine is electrolyzed in presence of catalase but the catalytic activity of the latter remains unchanged.

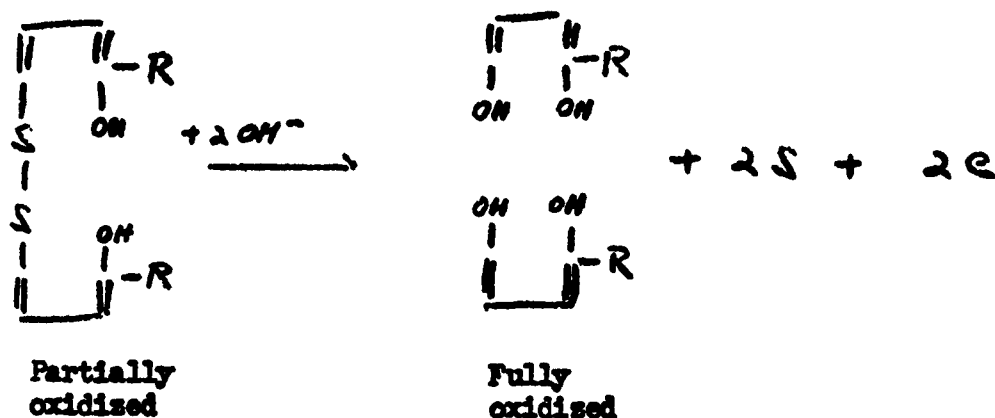
A great many details of enzymatic action have not been clarified, especially this is true in the case of catalase. The chemical linkage in the proteins that are responsible for the differentiation of the enzymatic activities cannot be very well applied to catalase; ferrihemoglobin forms catalytically inactive peroxide complexes while those of catalase show a tremendous activity.

When catalase is added to thienylalanine previously electrolyzed nothing is happened to catalase. In presence of thienylalanine it still shows a strong activity. We speculate that the oxidation that takes place in thienylalanine without catalase at the platinum anode is a partial oxidation involving with the thiophene ring; ultraviolet absorption band is noticeably suppressed, or displaces completely its characteristic peak, and this product of the initial oxidation is reducible at the dropping mercury cathode.

Speculation of the initial oxidation of thienylalanine:



When catalase is trapped in a permeable cellophane casing at anode, thienylalanine proceed to the second stage of oxidation as the ferric atom of catalase reduces to ferrous iron:



Catalase thus subjected to electrolysis in presence of thienylalanine is lost its catalytic activity. Fresh catalase is reducible at the mercury cathode but the catalase electrolyzed with thienylalanine does not show the reduction wave in polarograph, (graph 2 - January 31, 1952 report.)

STATUS OF RESEARCH

Objective:

The reversible inactivation of the catalytic action of catalase with thienylalanine, an antagonist of phenylalanine, by means of an electrolytic process.

General:

During the course of the study, it has been demonstrated in this laboratory that (1) the reversible inactivation of the catalytic action of catalase can occur in presence of thienylalanine when an electric energy is used, that (2) this is the first time to demonstrate the effect of an amino acid antagonist on another compound in vitro, that (3) this is the first to demonstrate that one form of inactivation of the catalytic action of catalase is caused by the reduction of its prosthetic iron, namely ferric to ferrous, and this demonstration offers an experimental proof to this particular problem which has been in discussion whether or not the oxidation-reduction system of the prosthetic groups involves with the catalytic activity, and that (4) this is the first time a pH sensitive organic compound, namely catalase, which has been so far impossible to withstand against electric current, is subjected without destroying the enzyme proper to an electrolytic process by means of a specially devised 4-electrode cell.

The first report, September 25, 1951, described the devise of the 4-electrode cell. Emphasis has been laid on the fact that the inactivation of catalytic activity of catalase results only when thienylalanine is added in this process. No inactivation has been observed in electrolysis when catalase alone is subjected. No inactivation occurs when catalase is electrolyzed in presence of phenylalanine. Thus the research has established the first step of the principle that the antagonist interferes with the catalase system while the counterpart has no affinity with the enzyme. The interference results in reduction of the prosthetic iron of catalase.

The second report, January 31, 1952, discussed the results of the catalase inactivation caused by analogous compounds to thienylalanine as well as sulfur containing amino acids. This report also contained the results of the preliminary polarographic analysis on a few compounds used as a reducing agent to catalase. The third report, September 1, 1952, discussed a further study of the improved technique of the 4-electrode cell which has shown more pronounced result than any of the former techniques. This report contained the discussion on anodic inactivation of catalase, experiments with platinised platinum plate, silver and copper anodes, the current efficiency, and the product of thienylalanine during the electrolysis.

Saburo Katsura
Research Associate.