STUDIES ON URICASE

FROM *NEUROSPORA CRASSA*

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1954

ACKNOWLEDGMENTS

I wish to thank Professor H. K. Mitchell for his continuous aid and counsel, and especially for setting an example of scientific conduct which I feel anyone would do well to follow.

I am indebted to Professor Sterling Emerson and Professor N. H. Horowitz for several helpful suggestions.

The financial assistance of the Atomic Energy Commission through a predoctoral fellowship administered by the Oak Ridge Institute of Nuclear Studies and of Mrs. Arthur McCallum and the Arthur McCallum Fellowship Fund is gratefully acknowledged.

ABSTRACT

A study has been made of the enzyme uricase from the mold Neurospora crassa.

It has been shown that the yield of the enzyme can be increased by growth of the mold in the presence of uric acid. It has also been observed that such addition of uric acid to the medium is stimulatory to growth.

A procedure has been devised for purification of this enzyme which is not bound to particulate material in the mold and a product has been obtained which has a higher specific activity than that of any uricase previously prepared. Neurospora uricase has been found to differ in some physical properties, such as solubility, and pH optimum, from the uricases prepared from animal sources. However, inhibition and activation experiments have shown that the enzymes from both sources are functionally similar.

Results of partial inhibition of uricase by p-chloromercuribenzoate and activation by reducing agents, such as glutathione and sulfide, have been obtained, which indicate involvement of sulfhydryl groups in at least an activating role.

A boiled extract of whole Neurospora has been found to contain at least one activating substance, and reasons have been given for believing that this activation is different from that produced by reducing agents.

The question of the existence of a uricase cofactor has been discussed.

Spectrophotometric evidence has been obtained for the existence of two or more transient intermediates during the course of uric acid oxidation. A product which has an absorption spectrum similar to those

observed for one or more of the transient intermediates has been prepared by oxidation of uric acid with alkaline permanganate.

The question of whether uricase is a single enzyme or a complex of enzymes has been discussed.

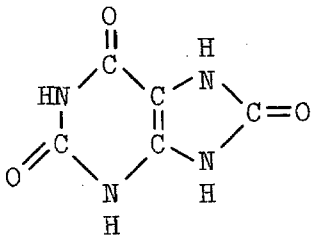
TABLE OF CONTENTS

I. INTRODUCTION

An enzyme system catalyzing the destruction of uric acid was shown to exist in various animal tissues by several workers near the turn of the century (1, 2, 3). Since this time uricase has been the subject of many extensive investigations, and yet because of the complexity of the reaction and the instability of the products, very little is known regarding the nature of the enzyme and the mechanism of its action. Uricase activity was first observed in Neurospora crassa by Mitchell and Haskins (4), and since the enzyme appeared to be non-particulate it was decided to investigate it further in order to find out more about the enzyme and the reaction, and to add to the biochemical characterization of Neurospora crassa.

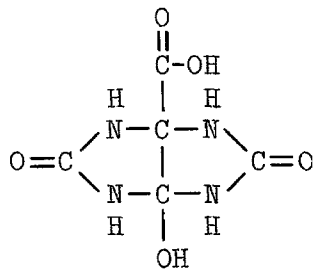
Figure 1

STRUCTURAL FORMULAE OF COMPOUNDS



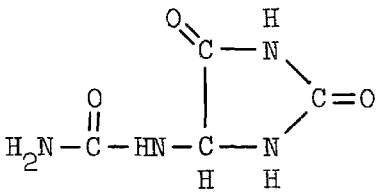
A Uric Acid

(2,6,8-trioxopurine)



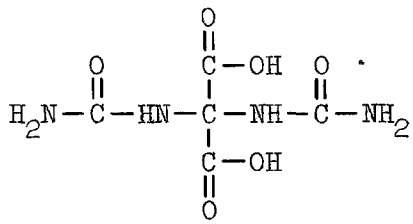
B Hydroxyacetylene-diureine-carboxylic Acid

(2,5,8-trihydroxy-tetrahydro-imidazo (d) imidazole-7-carboxylic acid)



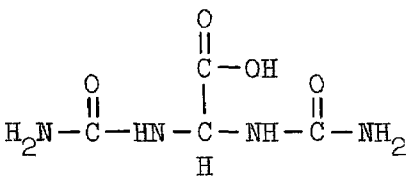
C Allantoin

(4(5) ureido hydantoin)



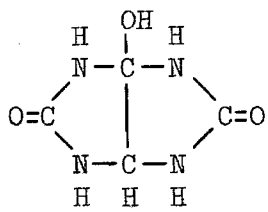
D Uroxanic Acid

(diureido malonic acid)



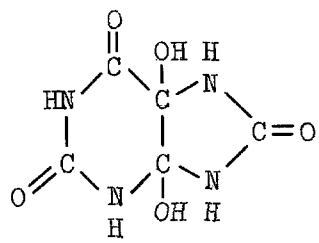
E Allantoic Acid

(diureido acetic acid)



F Isoallantoin

(2,5,7-trihydroxy-tetrahydro-imidazo (d) imidazole)



G Uric Acid-4,5-Glycol

II. SUMMARY OF PREVIOUS WORK

A. The Distribution of Uricase

Species Distribution. The presence or absence of uricase has been observed in many species of plants, animals and microorganisms. The distribution of the uricolytic enzymes (uricase, allantoinase, allantoicase and urease) in a few species is shown in Table I. Florkin and Duchâteau (5) suggested that the distribution of these enzymes in the animal kingdom has evolutionary significance, and proposed that the evolutionary process has been one of elimination of enzymes, with the higher forms having successively more complex end products of purine catabolism. Although these enzymes occur more frequently in the lower forms, there are enough exceptions to make any evolutionary sequence, based on this criterion, of questionable value. It seems unlikely that such an approach should be fruitful since the only apparent selective factor is one for retention of the enzymes by the lower forms which might have difficulty in the excretion of uric acid and allantoin which are much less soluble than the products of further catabolism.

Organ Distribution. Battelli and Stern (12, 13) have investigated the distribution of uricase among the various tissues of several mammals. They found that the richest sources of the enzyme are liver and kidney, with smaller amounts detectable in spleen. Muscle, lung, brain and pancreas were found to lack perceptible uricase activity. Such distribution studies have not been widely carried out in the lower species; however, the organ comparable to the liver has generally proven to be a good source.

Intracellular Distribution. In recent years, with the development

Table I*

DISTRIBUTION OF URICOLYTIC ENZYMES

Organism	Uricase	Allantoinase	Allantoicase	Urease	Reference
Corynebacterium renale	+	+**	..	+	6
Corynebacterium equi	-	-	..	-	6
Soja hispida (soy bean seed and seedling)	+	+	+	+	7
Hordeum vulgare (barley grain)	-	-	..	..	8
Basidiomycetes (several species)	+	+	..	..	9
Sipunculus	+	+	+	+	5
Frog	+	+	+	-	10, 11
Mammals other than primates	+	-	-	-	Numerous authors
Primates	-	-	-	-	Numerous authors
Lamperta	-	-	-	..	5
Earthworm	-	-	-	..	5

* The reactions catalyzed by these enzymes are uric acid (A - fig. 1) $\xrightarrow{\text{uricase}}$ allantoin (C - fig. 1) $\xrightarrow{\text{allantoinase}}$ allantoic acid (E - fig. 1) $\xrightarrow{\text{allantoicase}}$ glyoxylic acid + urea $\xrightarrow{\text{urease}}$ ammonia + carbon dioxide.

** Shown indirectly by NH_3 production from allantoin and by recovery of much smaller than stoichiometric quantities of allantoin after incubation with uric acid.

of special techniques, extensive investigations have been undertaken on the intracellular distribution of many enzymes. The first of these concerning uricase distribution was carried out by Lan (14) who isolated nuclei from rat liver by the method of Dounce (15) which employs repeated centrifugation with distilled water acidified to pH 6.0-6.2 with citric acid as the suspending medium. Lan reported that nuclei prepared in this manner have uricase concentrations 30 to 46 per cent higher than that of the whole homogenate; however, Dounce mentioned in his description of the method that these nuclei contain adsorbed hemoglobin so it is possible that other properties of the nuclei such as their uricase activity may be due to adsorbed protein. This interpretation seems especially attractive in the light of a later paper by Hogeboom, Schneider and Striebich (16) who prepared nuclei by homogenization of rat liver in 0.25 M sucrose - 0.00018 M calcium chloride, followed by layering of this material over 0.34 M sucrose - 0.00018 M calcium chloride and centrifugation of the nuclei from the less dense to the more dense layer. This layering-centrifugation procedure was repeated three times and the nuclei thus obtained were assayed for uricase activity. This preparation was found to contain an exceedingly small amount of uricase and by determining the homogeneity of the fraction using a Petroff-Hauser counting chamber, it was shown that the activity present could be accounted for by contamination with mitochondria and unbroken cells. A more complete study of this distribution was made by Schneider and Hogeboom (17) who fractionated mouse and rat liver homogenates using the more or less standard 0.88 M sucrose method and found 65.2 per cent of the activity in the mitochondrial and 32.4 per cent in the microsomal fractions. Similar results were shortly obtained by Schein, Podber and

Novikoff (18) who studied uricase distribution in rat liver homogenates and found 73 to 75 per cent of the activity in the mitochondrial fraction and 5 to 8 per cent in the microsomal fraction using either 0.88 M sucrose or distilled water as the suspending medium. Novikoff et al., (19) in a recent study of the heterogeneity of mitochondria and microsomes have been able to assign most of the uricase activity to the fractions containing only very small mitochondria and large optically dense microsomes. In all of these more complete distribution studies of the rat and mouse, activity in the soluble fraction has been very low or undetectable. On the other hand, Mitchell and Haskins (4) found in a similar study in Neurospora crassa that all of the uricase activity is in the soluble fraction. It is unfortunate that studies of this sort have not been carried out in a wider range of organisms, since knowledge of distributional differences of other enzymes, normally thought of as "particulate," if such differences exist, might help to outline the architectural requirements of the cell. However, this one case does serve as an example of a heritable difference with regard to subcellular organization of very similar enzymes.

B. The Purification of Uricase

Most of the early workers attempted purification of uricase using a variety of methods too numerous to mention here. These attempts were generally unsuccessful or were not pursued far enough to produce more than a few fold purification. The first highly purified uricase was prepared by Davidson (20) from pig liver using the procedure outlined in Figure 2. The enzyme obtained by this method is a white powder, soluble in alkaline borate buffer but insoluble in water or neutral solutions,

which on a weight basis catalyzes oxygen uptake 650 times as rapidly as the crude liver powder. Unfortunately catalase was not added to the reaction mixtures so that measurement of oxygen uptake gives spuriously high activity values when the catalase of the crude preparation is removed. A short time later Holmberg (21) published a modification of this procedure which is outlined in Figure 3. The product of this fractionation has a QO_2 of 6000 in the presence of added catalase as opposed to one of 5 in the crude liver powder and therefore appears to be more highly purified than that of Davidson. The appearance and solubility properties of the two enzymes are very similar. Davidson (22) later published a comparison of the two methods and stated that the specific activities of both of the products were the same.

One of the greatest limitations of the enzymes prepared by these methods is their insolubility, and they must often be used as suspensions. Therefore Leone (23) has recently devised a method for the partial purification of uricase from beef kidney, which is designed to give a stable, soluble product in relatively high yield. This procedure is outlined in Figure 4. Possible reasons for the increased solubility of this product are the autolysis of the homogenate during extraction of the enzyme; precipitation of extraneous material by dialysis against 0.01 M borate buffer; or perhaps just because the enzyme from beef kidney is more soluble than that from pig liver.

The first steps of all these procedures are unsuitable for the fractionation of uricase from Neurospora which is soluble, since they are designed for use with particulate enzymes. Other differences between mammalian and Neurospora uricases, which would prevent direct use of any of the above schemes, are the destruction of crude Neurospora uricase

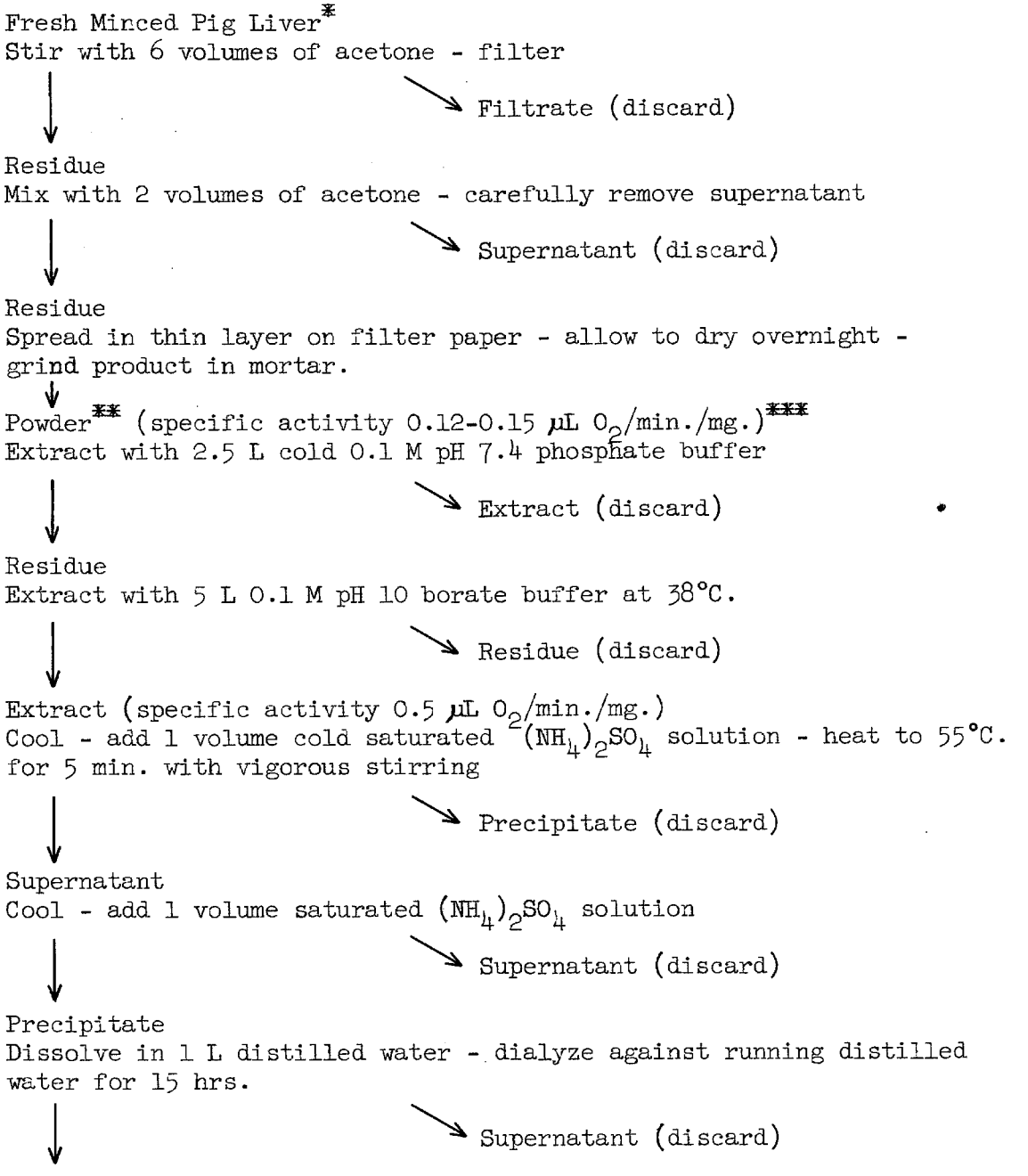
by gentle heating techniques or prolonged dialysis, and the enzyme's solubility in neutral or even acidic solutions. The adsorption techniques were not used since it had been observed that Neurospora uricase tends to adsorb incompletely onto gel-like precipitates and to be extracted only with great difficulty. In general, it appears that the properties of the two enzymes are different enough so that each step in a mammalian fractionation scheme would have to be carefully tested and modified before it could be used in purification of the Neurospora enzyme. With regard to the value of Neurospora crassa as a source of uricase, it has been observed that the mold grown in the presence of uric acid has an enzyme concentration of the same order of magnitude as that found by Klemperer (24) in fresh dog liver.

C. The Properties of Uricase

It appears from the results of very extensive studies that uricase exhibits absolute specificity for both substrate and electron recipient. It shows no activity toward any of a large number of similar compounds which have been tested, such as xanthine, hypoxanthine, hydroquinone (25), uric acid 4,5-glycol (26), 8-azaguanine, 8-azaxanthine (27), uric acid riboside (28), all the possible mono-, di-, tri- and tetra-methyl uric acids and several of the ethyl uric acids (29). In fact most of these compounds competitively inhibit the reaction to some extent. The effects of some of these inhibitors are shown in Table II. Electron recipients such as methylene blue (25, 30), hydrogen peroxide and ethyl hydroperoxide (13) fail to react in this system.

A large number of structurally unrelated compounds have been tested for inhibition or activation of the enzyme in order to gain some insight into the nature of the active groups and the prosthetic group if one

Figure 2



* Acetone Powder prepared according to Keilin and Hartree (29).

** All quantities of reagents are those used for treatment of 250 g. of acetone powder.

*** Incubated in atmosphere of O_2 without added catalase.

Figure 2 (cont.)

↓

Precipitate
Wash with 400 ml. 0.1 M pH 7.4 phosphate buffer

↓ → Supernatant (discard)

Residue
Extract with 2 250 ml. volumes 0.1 M pH 10 borate buffer

↓ → Residue (discard)

Extract (specific activity 10-25 μL O_2/min./mg.)
Add 1 volume saturated $(NH_4)_2SO_4$ solution

↓ → Supernatant (discard)

Precipitate
Dissolve in 60 ml. cold water - add justenough acetone to cause a flocculant precipitate after standing 20 min. (0.5-1.0 volume)

↓ → Precipitate (discard)

Supernatant
Remove acetone by vacuum distillation at 20°C. (after adding 2 drops of octyl alcohol) - allow to stand overnight - a precipitate slowly forms

↓ → Supernatant (discard)

Precipitate
Wash 3 times with small amounts of distilled water - extract with 5 ml. 0.1 M pH 10 borate buffer

↓ → Residue (discard)

Supernatant (specific activity 85-90 μL O_2/min./mg.)
Final Product. Enzyme can be precipitated by dialysis.

Figure 3

Procedure same as that of Davidson (fig. 2) through extraction of acetone powder with borate buffer. All quantities of reagents are those used for treatment of 200 g. of acetone powder (QO_2 of acetone powder = 5).*

↓

Extract

Add 25-50 g. $Ba(OAc)_2$

↓ → Precipitate (discard)

Supernatant

Remove excess barium with $(NH_4)_2SO_4$ - add 1 volume saturated $(NH_4)_2SO_4$ solution.

↓ → Supernatant (discard)

Precipitate

Dissolve in enough distilled water to give solution about 0.1 saturated in $(NH_4)_2SO_4$ - heat to 55-60°C. for 5 min.

↓ → Precipitate (discard)

Supernatant

Add 40 g. of $Ba(OAc)_2$

↓ → Precipitate (discard)

Supernatant

Repeat above $Ba(OAc)_2$ treatment

↓ → Precipitate (discard)

Supernatant

Adjust to pH 7 - add 50 ml. alumina C-γ (2.0 g.)

↓ → Alumina C-γ (discard)

Supernatant

Add 1 volume of saturated $(NH_4)_2SO_4$ solution (neutralized with NaOH)

↓ → Supernatant (discard)

Precipitate

Extract with 200 ml. distilled water - filter rapidly

↓ → Residue (discard)

cont.

---

* Incubated in air with added catalase.

Figure 3 (cont.)

↓

Filtrate

Allow to stand 1-2 hrs. - a fine white precipitate forms

↘ Supernatant (discard)

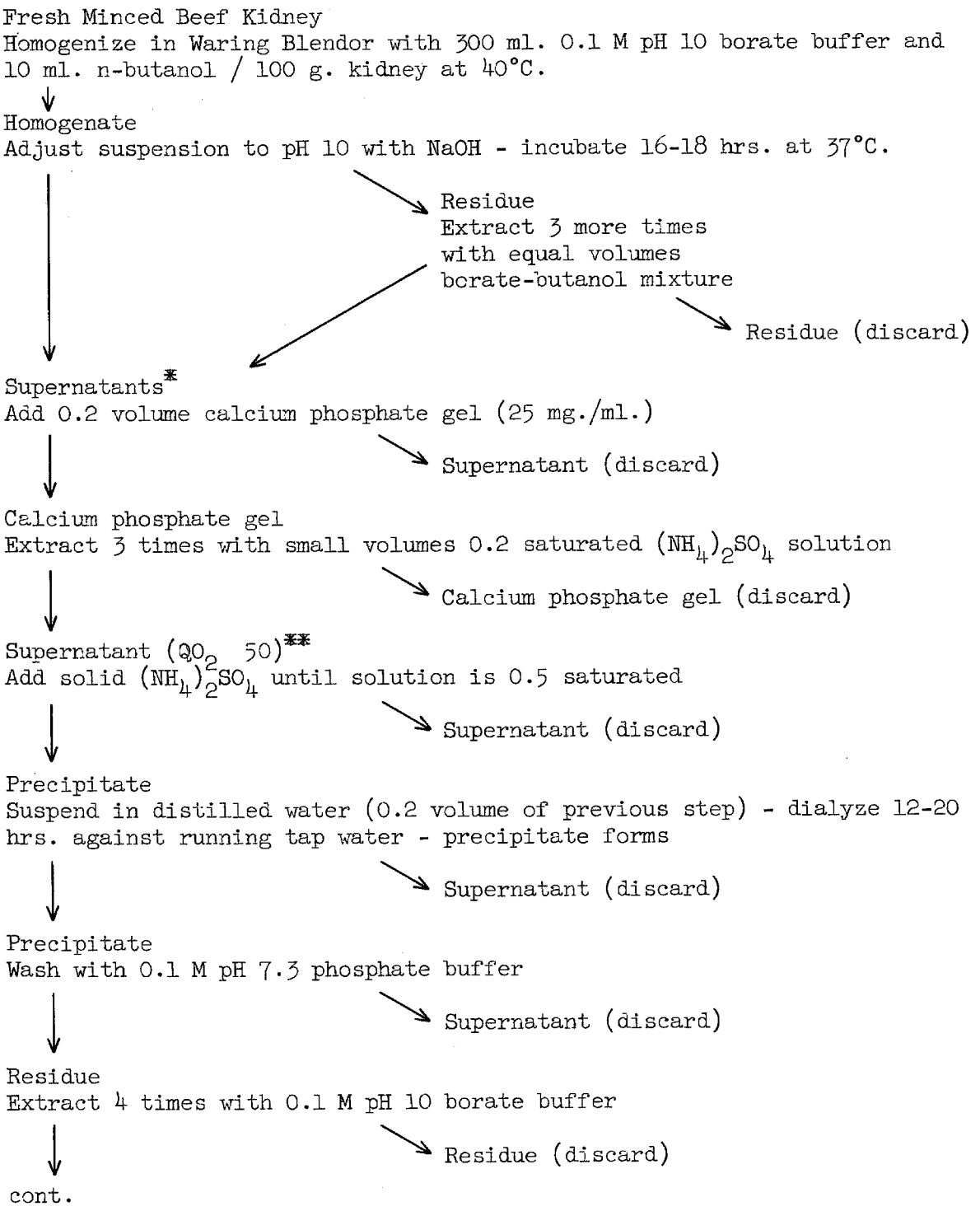
↓

Precipitate

Wash twice with small amounts of distilled water

This is the "purified uricase" (QO_2 6000)

Figure 4



* No catalase detectable at this stage.

** Incubated in air without added catalase.

Figure 4 (cont.)

↓

Extract (QO_2 300)
Acidify to methyl red end point - add 0.1 volume calcium phosphate gel

↘ Supernatant (discard)

↓

Calcium phosphate gel
Wash with distilled water - extract 3-4 times with neutral or slightly alkaline 0.2 saturated $(NH_4)_2SO_4$ solution

↘ Calcium phosphate gel (discard)

↓

Extract (QO_2 700)
Make 0.5 saturated with $(NH_4)_2SO_4$

↘ Supernatant (discard)

↓

Precipitate
Wash with 0.5 saturated $(NH_4)_2SO_4$ solution - dissolve in 0.1 M pH 10 borate buffer

↓

Solution
Dialyze 36 hrs. against 3 changes of 0.01 M pH10 borate buffer - precipitate forms

↘ Precipitate (discard)

↓

Supernatant (QO_2 750-800)
Final Product

Table II

EFFECTS OF SOME COMPETITIVE INHIBITORS ON URICASE

Inhibitor	Mole Ratio (Inhibitor : Uric Acid)	% Inhibition	Reference
1,3,7-trimethyl-uric acid	1	68	29
1-methyluric acid	1	58	29
3-methyluric acid	1	47	29
7-methyluric acid	1	40	29
1,3,9-trimethyl-uric acid	1	16	29
8-azaguanine	8.22×10^{-3}	26	27
8-azaxanthine	1.49×10^{-4}	62	27

exists. The effects of some of the inhibitors are shown in Table III. Slight activation effects are given by a variety of sulfhydryl compounds such as thioacetic acid (22) and cysteine (31), with glutathione (31) activating to a considerably higher level. Various chelating agents such as pyrophosphate, α,α'-dipyridyl and dithizone have been reported to cause a slight activation by some workers (22, 29) and to have no effect by others (21). The rate of the reaction is not affected by 0.1 M sodium fluoride, 5 per cent urethane, 0.004 M sodium sulfide (29), sulfanilamide, carbon monoxide ($CO:O_2$ = 49:1, inhibition tested in the dark) (22), or 0.0012 M BAL (32). Contrary to the results of Keilin and Hartree (29), shown in Table III, Holmberg (21) reported that azide has only a transitory inhibition at pH 7 which disappears in five minutes. Davidson (22) reported that thiocyanate, which inhibits carbonic anhydrase by supposedly complexing the zinc, incompletely inhibits uricase at high concentrations (up to 0.1 M) while Wachtel et al. (33) reported that 0.022 M thiocyanate has no effect. Several workers (21, 22) have found that uricase is destroyed by hydrogen peroxide, yet Stadie and Haugaard (34), working with rat tissue slices and homogenates, reported that storage under seven atmospheres of oxygen for four hours has no effect on uricase. Davidson (20) and Holmberg (21) examined their purified enzymes spectroscopically but were unable to detect any adsorption bands in the visible range. Finally the purified enzyme preparations have been analyzed for heavy metals with conflicting results. Davidson (20) reported that his purest enzyme contained a relatively large amount of iron (0.15 - 0.20 per cent) and that during the last stages of preparation increase in iron content paralleled increase in enzyme activity. Shortly thereafter, Holmberg (21) found that his purest enzyme contained

Table III

EFFECTS OF SOME INHIBITORS ON URICASE

Inhibitor	Concentration M/L	% Inhibition	Reference
KCN	0.001	94	29
KCN	0.0001	83	29
NaN_3 (pH 8)	0.002	28	29
NaN_3 (pH 6.8)	0.002	40	29
$NH_2OH \cdot HCl$	0.02	45	29
8-hydroxyquinoline	0.004	10-14	29
$K_4Fe(CN)_6$	0.015	10	22
Cu	0.00002	75	22
Fe	0.001	35	22
Mn	0.001	35	22
Zn	0.002	80	22
Co	0.001	60	22
Ni	0.001	40	22

a much smaller amount of iron (0.025 per cent) but contained a relatively large amount of zinc (0.13 per cent); analyses for lead were negative. Subsequently Davidson (22) in a paper comparing the two purification procedures found 0.15 - 0.20 per cent iron and only 0.09 per cent zinc in both enzymes. He also performed spectroscopic analyses for other heavy metals and found traces of copper, lead, manganese, magnesium, aluminum and tin, but none of these was present in concentrations high enough to be considered important. Recently Praetorius (32) dialyzed uricase exhaustively against solutions of BAL and was unable to detect any zinc when the treated enzyme was analyzed in a quartz spectrograph, although the enzyme was fully active. According to Pratorius' calculations, 0.04 per cent zinc could have been detected by his analytical technique and therefore he considers this value to be the maximum zinc concentration.

Unfortunately this rather miscellaneous collection of facts does not present a clear picture as to the nature of the active groups of uricase. The most consistent observation is the high degree of reversible cyanide inhibition in low concentrations which indicates participation of a heavy metal ion (21, 22, 25, 29, 30). However, the inactivity of the various chelating agents and the conflicting results obtained with azide do not lend support to this hypothesis; the analytical results are not consistent enough to tell which metal ion is most likely to be involved. The reports of activation by chelating agents are probably due to relief of heavy metal inhibitions. The results of activation by sulfhydryl compounds, destruction by hydrogen peroxide and heavy metal inhibition might indicate that sulfhydryl groups are required for activity, but definitive experiments have not been done.

D. The Mechanism of Uric Acid Oxidation

The first study of the mechanism of uric acid oxidation was done by Wiechowski (35) who isolated allantoin in stoichiometric quantities from the reaction mixtures after uric acid destruction. Shortly after the work of Wiechowski, Battelli and Stern (13, 36) studied the gas exchange of the reaction and found that it had an RQ of two when fresh extracts were used, a result which would be expected with a stoichiometric conversion of uric acid to allantoin by an enzyme preparation containing catalase. These results were later confirmed by Grynberg (37) and by Keilin and Hartree (29). All these workers used crude enzyme preparations and they all observed that, as the enzyme aged, the RQ became lower. The reason for this decrease in RQ was explained by Keilin and Hartree who showed that hydrogen peroxide was produced in the reaction and that in aged preparations coupled oxidations of other substrates were occurring by utilization of hydrogen peroxide. Davidson (22), working with a highly purified enzyme preparation, obtained an RQ of one with uricase alone and an RQ of two in the presence of catalase. He also isolated and identified allantoin from the reaction mixture but he did not estimate the yield of this product. From all these data it appeared that allantoin is the only product of uricase action and that the oxidation of uric acid is a straightforward reaction catalyzed by a single enzyme. Nevertheless, more than one step seemed necessary and Grynberg (37) postulated the formation of uric acid 4,5-glycol (G - fig. 1) as an intermediate product in allantoin formation, but Brünig et al. (26) were unable to get carbon dioxide or allantoin by incubation of this compound with uricase.

Complicating factors arose from the work of Felix, Scheel and Schuler (38), who, using an acetone powder from pig liver, showed that

the amounts of carbon dioxide produced during the reaction could be changed by altering experimental conditions. In some of their experiments the yields of carbon dioxide were as low as 18 per cent of the theoretical. They also demonstrated that their uricase preparation exhibited two pH optima (8.9 and 9.9) for the destruction of uric acid, but only one (9.9) for the production of carbon dioxide. They concluded from these results that the uricase reaction is catalyzed by two enzymes, a urico-oxidase which yields an unknown product and a decarboxylase which converts this product to allantoin. In support of this hypothesis they stated that an extract made with glycerol - pH 8.9 phosphate buffer was relatively rich in oxidizing and relatively poor in decarboxylating activity. Unfortunately no data were given to back this statement. The observation of two pH optima was confirmed by Kleinmann and Bork (39), but Keilin and Hartree (29) were unable to obtain these results, although both groups of workers used acetone powders from pig liver, prepared in similar ways, as their enzyme sources.

In a second paper, Schuler (40) reported inability to separate the postulated two enzymes to any workable extent, or to isolate the intermediate product from reaction mixtures. However, on the basis of observations made on the crude reaction mixtures after oxidation with uricase, Schuler listed the following properties which he considered must be characteristic of the intermediate compound.

1. It must be a stronger acid than uric acid.
2. It must split off carbon dioxide easily in acid solution.
3. It must yield urea after treatment for some time with strong acetic acid.
4. It must give the Fosse-Bossuyt reaction for allantoin (color reaction for glyoxylic acid after hydrolysis).

Felix, Scheel and Schuler (38) tested uroxanic acid (D - fig. 1) as an intermediate but no carbon dioxide or allantoin was formed when this compound was incubated with uricase. In an immediately following paper Schuler and Reindel (41) reported the preparation of hydroxyacetylene-diureine-carboxylic acid (B - fig. 1) by alkaline permanganate oxidation of uric acid in the cold. The properties of this compound are in keeping with those expected for the proposed reaction intermediate. Expanding on this point in a still later paper (42), Schuler and Reindel described studies of the oxidation of uric acid by various reagents and found that only two of the treatments investigated resulted in the conversion of uric acid to allantoin. The treatments producing allantoin are: oxidation of uric acid in alkaline solution with stoichiometric amounts of permanganate, and catalytic oxidation of uric acid in alkaline solution with oxygen and manganese dioxide. They found further that by making the conditions mild, B (fig. 1) could be obtained in high yield by use of these reagents. An aliquot of the reaction mixture, after catalytic oxidation of uric acid with oxygen and manganese dioxide under mild conditions, was acidified to pH 9 and incubated with uricase. Carbon dioxide was produced in amounts comparable to those obtained during the enzymatic oxidation of an equivalent amount of uric acid. By silver precipitation of the deproteinized reaction mixture, after enzymatic oxidation of uric acid, they were able to isolate a silver salt which, although not analytically pure, had the same general properties of the trisilver salt of B (fig. 1). Finally they pointed out the importance of the fact that the allantoin obtained from the enzyme catalyzed reaction is racemic, a finding which suggests that the last product of enzyme action must be symmetrical. This suggestion has recently been supported

by the work of Brown et al. (43, 44) who fed uric acid and adenine labeled with N^{15} in the one and three positions to rats and found that the allantoin isolated from the urine was evenly labeled in all four nitrogen atoms. With regard to destruction of intermediates similar to B (fig. 1), Schuler and Reindel (42) hypothesized that a decarboxylating enzyme, if one exists, must act before ring opening in order to yield racemic allantoin. They could find no evidence for intermediate production of isoallantoin (F - fig. 1) and therefore presumed that decarboxylation and ring opening occur almost simultaneously.

Klemperer (45) studied uric acid oxidation manometrically using a partially purified uricase preparation, and found that the amount of carbon dioxide production is limited at low pH values or in the presence of borate. He also found that no further carbon dioxide evolution occurs after the oxygen uptake is complete. After carrying out the reactions in manometers and estimating allantoin from carbon dioxide production he analyzed the reaction mixtures by a micro Van Slyke amino-nitrogen technique. This reaction releases as nitrogen any amino groups in open chains but does not affect those incorporated into stable rings. For example, uric acid gives no nitrogen, allantoin and B (fig. 1) two equivalents, and uroxanic acid four equivalents of nitrogen when treated in this manner. When the enzymatic reaction mixtures were assayed in this way and the resulting nitrogen values corrected for allantoin, it was found that the products had between two and four equivalents of labile nitrogen depending upon the conditions of enzyme incubation. Klemperer interpreted these data as meaning that B (fig. 1) and uroxanic acid are present in the final reaction mixture in varying proportions. He also concluded that neither of these compounds is an intermediate since there

is no further liberation of carbon dioxide after oxygen uptake is complete. In opposition to the hypothesis of Felix, Scheel and Schuler (38) he proposed that uricase is a single enzyme catalyzing the oxidation of uric acid, with the product decomposing spontaneously according to the following scheme.

$$\text{uric acid} + H_2O + O_2 \xrightarrow{\text{uricase}} C_5N_4O_4H_4 + H_2O_2$$

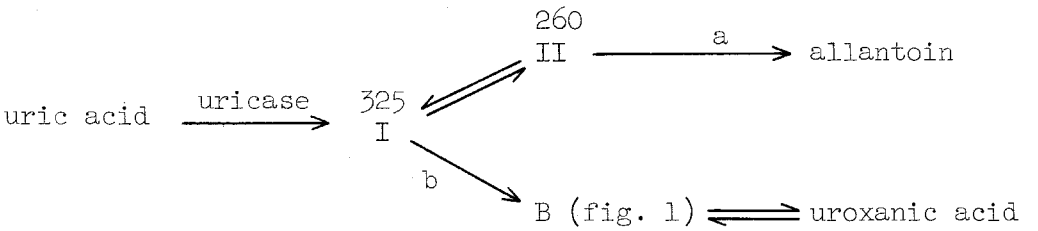
$$C_5N_4O_4H_4 \xrightarrow{H_2O} \text{allantoin} + CO_2$$

$$C_5N_4O_4H_4 \xrightarrow{H_2O} \text{B (fig. 1)}$$

$$C_5N_4O_4H_4 \xrightarrow[\text{spontaneous}]{2H_2O} \text{uroxanic acid}$$

An additional contribution to this picture was made by Praetorius (46), who made use of a highly purified uricase prepared according to Holmberg (21). Evidence was obtained for the existence of at least two intermediates, one which absorbs at shorter wavelengths and was measured at 260 mμ [II] (a minimum in the uric acid absorption spectrum) and one which absorbs at longer wavelengths and was measured at 325 mμ [I] (a longer wavelength than those of the uric acid absorption spectrum). Evidence was given for the presence of a third intermediate absorbing at 293 mμ, but this was not studied intensively and its existence is not well established. [I] is extremely unstable and its decomposition is accelerated by high pH and by borate ions; it disappears very rapidly when alkali or borate is added to the reaction mixture. The rate of its formation follows closely the rate of uric acid destruction and at a given pH it responds very rapidly and in the expected way to conditions which alter the rate of uric acid destruction. In time, [II] appears very shortly after the appearance of [I], and requires a longer time to be broken down, still being detectable after all the uric acid and [I] have disappeared. The accumulation of this intermediate is also decreased

in the presence of borate, but addition of alkali to the reaction mixture results in a very rapid rise in absorption at 260 mμ, indicating that [I] is probably being converted into [II] by this treatment. From these data and those of Klemperer (45), Praetorius devised the following scheme.



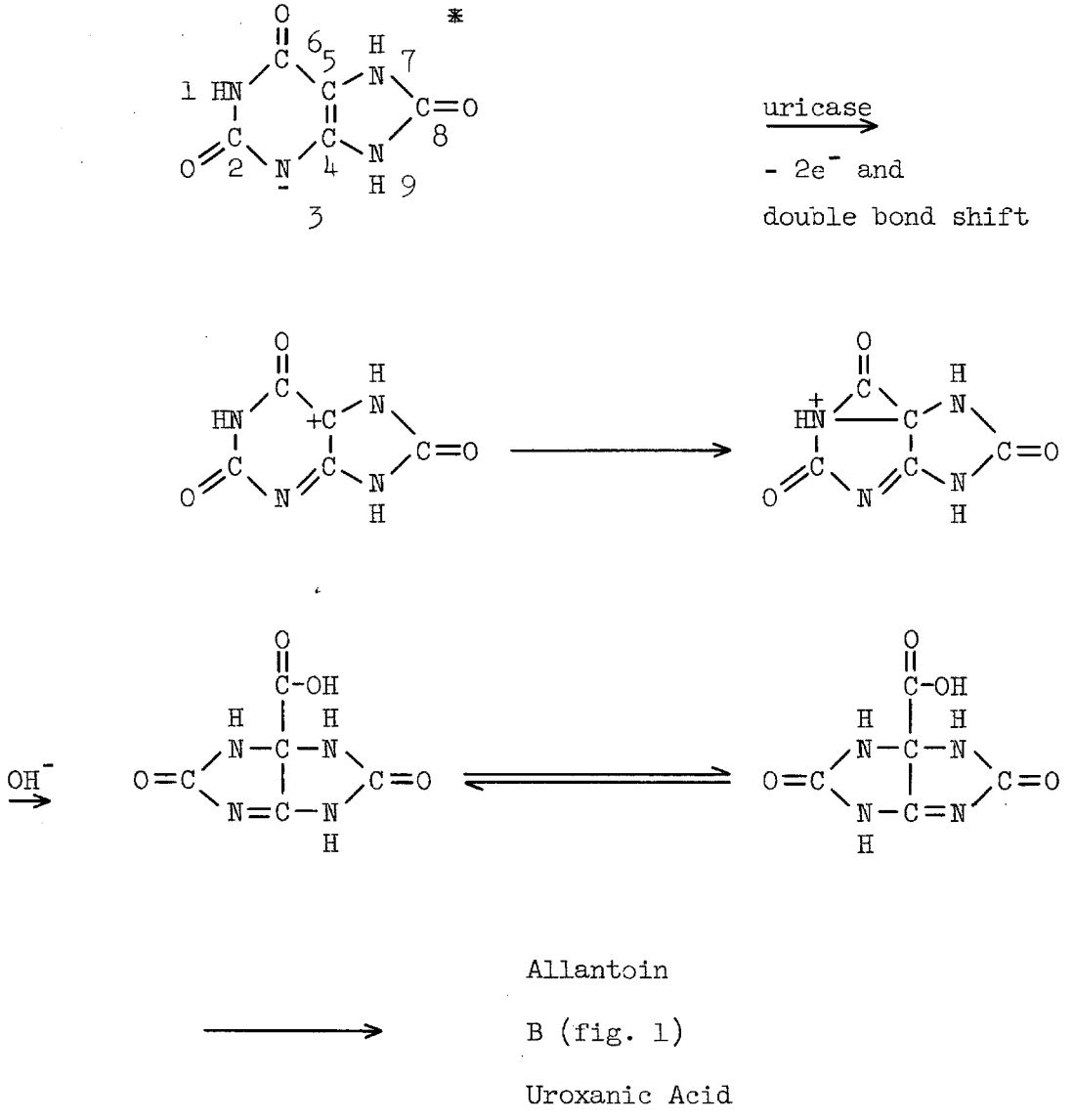
a is accelerated at a high pH

b is accelerated by borate

Bentley and Neuberger (47) have shown from tracer studies using a partially purified uricase that the oxygen of the hydrogen peroxide comes from molecular oxygen rather than from water, which may indicate a rather direct transfer of electrons from uric acid to oxygen. They have also shown by use of 6-C^{14} uric acid that the carbon dioxide comes from carbon 6 of uric acid which is consistent with the hypothesis that B (fig. 1) or a similar compound is an intermediate in the decomposition. They assigned to uricase the function of transferring two electrons from the mono- or di-anion of uric acid to oxygen with a subsequent spontaneous decomposition of the oxidation product according to Figure 5. They stated that the tautomerism of the unsaturated compound is fast enough to fulfill the requirement of a symmetrical intermediate without necessitating conversion to B (fig. 1). A possible mechanism for using the energy of this reaction has been suggested by Bentley and Neuberger who point out that a high energy phosphate bond would be obtained if the three-membered ring were cleaved by phosphorolysis rather than hydrolysis.

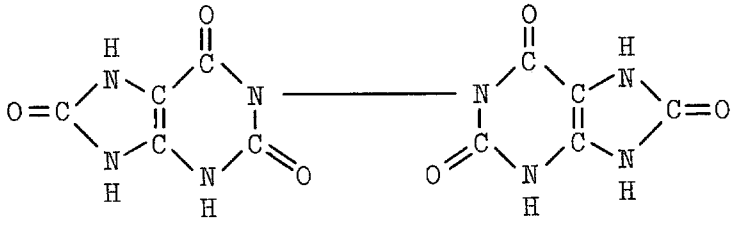
Figure 5

PROPOSED REACTION MECHANISM OF BENTLEY AND NEUBERGER (47)



* Other contributing ions are those with the charge in the 9 and 5 positions.

A further speculation as to the nature of the primary product of uric acid oxidation has been made by Griffiths (48) who studied the oxidation of uric acid in alkaline solution with catalysis by 10^{-4} M cupric ion. During the course of this reaction, transient absorption was observed at 325 mμ and upon further investigation it was found that the material absorbing at this wavelength had properties very similar to compound I of Praetorius (46). It was also noticed that about 0.25 mole of carbon dioxide was produced per mole of uric acid oxidized, a value which is consistent with some of the results of Klemperer (45). Therefore Griffiths believed that the copper catalyzed reaction is similar to that catalyzed by uricase, and, by analogy with the copper catalyzed oxidation of cysteine and ascorbic acid, postulated that a "dehydro uric acid" is the first product. Without further evidence he suggested that a possible structure for "dehydro uric acid" might be the one given below.



Although there is little evidence for such a structure, it might be said that this compound would be more likely to absorb in the 325 mμ region than any of those suggested by Bently and Neuberger (47).

E. The Status of the Problem

It seems likely that the more highly purified uricases which have been studied contain only one enzyme catalyzing uric acid destruction and that the complexity of the reaction is due to the spontaneous

decomposition of the primary oxidation product to a variety of compounds, depending upon the conditions. However, since these enzymes were all obtained by disruption of mitochondria it is possible that they might be stable complexes of more than one enzyme and it would be desirable to continue purification work in an attempt to obtain an enzyme which is well characterized, if not homogeneous. It should be remembered that even if studies of this nature show that a single enzyme is capable of catalyzing the reaction, the existence of an enzyme which catalyzes the decomposition of the primary reaction product to yield allantoin has not been ruled out. Studies on the course of the reaction have yielded evidence for the existence of at least two transient intermediates and several speculations have been made regarding the identity of these compounds. Although identification of the intermediates will be difficult because of their instability it might be possible to prepare more stable derivatives from the crude reaction mixture or, by the use of special reagents, to cause formation of abnormal products which might be characteristic of a given intermediate. Finally the state of knowledge about the active groups of uricase is highly unsatisfactory and should be investigated further with a purer enzyme. Knowledge about the prosthetic group of uricase, if any, might give information regarding biological utilizability of the energy released by this oxidation.

III. MATERIALS AND METHODS

A. Strain

All the experiments described in this work were carried out using Neurospora crassa wild strain 5297a. However, estimates of uricase activity have been carried out occasionally on crude extracts of other wild strains of Neurospora and comparable quantities of uricase have been found.

B. Growth of Mold

Neurospora, for use in large scale enzyme fractionation experiments, was grown in 15 L. carboys containing Fries minimal medium (49) supplemented with uric acid in a concentration of 2×10^{-3}M. For inoculation of the carboys, cultures of 5297a were grown on 15 ml. of Fries minimal - 3 per cent agar medium in 125 ml. Erlenmyer flasks until conidiation was heavy. Conidia from ten of these flasks were suspended in sterile distilled water and used to inoculate one carboy. The carboys were then incubated at 25°C. with aeration for two days. Mold was harvested by filtration through cheesecloth, washed twice with approximately 500 ml. distilled water and squeezed as dry as possible. Mold was then stored in the deep freeze until needed. The average yield was 400-500 grams of mold per carboy. Other media and growth conditions used in specific experiments are outlined in the descriptions of those experiments.

C. Determination of Enzyme Activity

Activity determinations were carried out by incubating the enzyme preparation to be assayed with uric acid and measuring the decrease in optical density at the uric acid absorption maximum with the Beckman

model DU quartz spectrophotometer. For qualitative or rough semi-quantitative assay an initial uric acid concentration of 20 γ/ml. was used and the incubation mixtures were read directly at 293 mμ (absorption maximum of uric acid in the pH 8-9 region). This method is very sensitive but does not give quantitative results since the rate is limited by the low uric acid concentration. For the purpose of quantitative estimation of uricase activity, a uric acid concentration of 200 γ/ml. was used (100 γ/ml. in some early experiments), and incubation was carried out in 0.1 M tris-hydroxymethyl-amino-methane buffer solution (1:1 borate-pyrophosphate used in early experiments) at 35°C. with shaking. Samples were taken from the incubation mixture from time to time and diluted ten fold with 0.17 N hydrochloric acid which neutralizes the buffer and gives a final pH of approximately one. Uric acid concentrations were determined at 285 mμ (the absorption maximum of uric acid at pH 1) using as the blank a sample from a reaction mixture containing all the constituents except uric acid. Under these conditions the amount of uric acid broken down during a given interval is directly proportional to the enzyme concentration as is shown below (a solution containing 20 γ/ml. has an optical density of 1.40).

Volume of enzyme	Decrease in optical density		
solution in ml.	20 min.	40 min.	60 min.
0.2	0.61	1.04	1.29
0.1	0.31	0.51	0.64

It will be noted from the above data that, although the amount of uric acid destroyed during a given interval is proportional to the uricase concentration, the rate decreases as time passes. Evidence that the rate is zero order and that this decrease is due to inactivation of the enzyme during the course of the reaction, has been given by experiments

on glutathione activation. A typical result is given below.

Additive	Decrease in optical density	
	20 min.	40 min.
None	0.41	0.68
GSH	0.56	1.10

The fact that the inactivation of the enzyme is stopped by addition of a reducing agent suggests that this inactivation is due to an oxidation, probably by the hydrogen peroxide which is produced during the reaction.

Thus it can be seen that good quantitative results can be obtained by use of this assay method. However for precise results, comparison of dilutions necessary to give a standard activity might be a better method.

D. Preparation of the Enzyme

The procedure that has been devised for purification of Neurospora uricase is outlined in Figure 6. Material from 5.5 kg. of Neurospora (wet weight) was fractionated by the first steps of this procedure, but since fairly large quantities were used to test subsequent steps and for various other experiments, it was not all carried through the entire scheme.

Comments on the purification procedure. The technique of pulverizing the frozen mold in a Waring Blendor which had been cooled with dry ice was suggested by Professor Sterling Emerson. By use of this method large amounts of material can be ground to a fine powder with relative ease.

It is not known whether the use of activated charcoal during the preparation of the crude extract contributes to the purification of the enzyme; however, this step was necessary to remove light absorbing substances so that the crude extract could be assayed in the spectrophotometer.

Figure 6

Mold
Freeze with dry ice - break into chunks - pulverize in Waring Blendor cooled with dry ice

↓

Fine powder*
Thaw - mix with Norit A (3 g./100 g. mold) - centrifuge at 20,000 x g for 1 hr.

→ Residue
Suspend in sufficient distilled water to make total volume 1 ml./1 g. mold - centrifuge at 20,000 x g for 1 hr.

→ Residue (discard)

↓

Supernatant (Relative specific activity 1)
Add 0.05 M K_2HPO_4/L (pH 7.2) - add 0.9 volume of acetone - allow to stand 8 hrs. - centrifuge at 2,500 x g for 5 min.

→ Precipitate
Wash with 0.1 volume 45% acetone - centrifuge at 2,500 x g for 5 min.

→ Precipitate (discard)

↓

Supernatant
Add 0.1 volume acetone - allow to stand 4 hrs. - centrifuge at 2,500 x g for 5 min.

→ Precipitate
Wash with small volume 50% acetone - centrifuge at 2,500 x g for 5 min.

→ Precipitate (discard)

↓

Supernatant
Adjust to pH 5.5 with citric acid - add 0.11 volume acetone - allow to stand 8 hrs. - centrifuge at 2,500 x g for 5 min.

→ Supernatant (discard)

↓

cont.

---

* All work after thawing of mold was carried out in the cold room at 2 - 4°C.

Figure 6 (cont.)

↓

Precipitate
Extract repeatedly with 0.1 M pH 7.2 phosphate buffer and distilled water until residue has negligible activity (Extraction - suspend in 1 volume buffer - centrifuge at 2,500 x g for 30 min. - suspend in 0.5 volume buffer - centrifuge at 2,500 x g for 1 hr. - suspend in 0.5 volume buffer - centrifuge at 20,000 x g for 1 hr. - suspend in 0.5 volume water - centrifuge at 20,000 x g for 1 hr. - suspend in 0.5 volume water - centrifuge at 20,000 x g for 1 hr.)

↘ Residue (discard) (contains less than 1% of activity)

↓

Extract (Relative specific activity 10-15)
Add 0.1 volume boiled extract (Section III-E) - Add 1 volume saturated $(NH_4)_2SO_4$ solution adjusted to pH 7 - allow to stand 3 hrs. - centrifuge at 600 x g for 10 min.

↘ Supernatant (discard)

↓

Precipitate
Suspend in 0.5 volume distilled water - reprecipitate by addition 0.5 volume saturated $(NH_4)_2SO_4$ solution - allow to stand 30 min. - centrifuge at 600 x g for 10 min.

↘ Supernatant (discard)

↓

Precipitate
Suspend in 1 volume distilled water - dialyze against distilled water 4 hrs. - centrifuge at 600 x g for 10 min.

↘ Precipitate (discard)

↓

Solution (Relative specific activity 25-30)
Adjust to pH 5.0 with HCl - centrifuge at 600 x g for 10 min.

↘ Precipitate (discard)

↓

Supernatant
Adjust to pH 4.5 with HCl - centrifuge at 600 x g for 10 min.

↘ Precipitate (discard)

↓

Supernatant (Relative specific activity 50-60)
Adjust to pH 7.0 with NaOH - make 0.1 M in pH 7 phosphate buffer - add 1.2 volumes acetone - allow to stand for 3 hrs. - centrifuge at 600 x g for 10 min.

↘ Supernatant (discard)

↓

cont.

Figure 6 (cont.)

↓

Precipitate
Extract twice with 2 volumes water - centrifuge at 20,000 x g for 30 min.

↓ ↘ Residue (discard)

Extract (Relative specific activity 100)
Dialyze against 0.04 M pH 8.2 tris-hydroxymethyl-amino-methane buffer - fractionate by sponge electrophoresis

↓ ↘ Inactive fractions (discard)

Active Fractions. (Relative specific activity 400)
Final Product.

The uricase in crude extracts exhibited varying degrees of stability, and in some cases all the activity disappeared after storage at 4°C. overnight. This instability is probably due to the presence of proteolytic enzymes. After investigation of several stabilizing techniques and quick fractionations designed to separate the uricase from the proteases, it was found that addition of acetone stopped the uricase destruction. This finding necessitated the use of acetone during the first step of fractionation although it is possible that other solvents might have the same effect.

Uricase shows a tendency to coprecipitate when proteins or gel-like materials are precipitated in its presence, especially under conditions near to those which precipitate uricase. When uricase is coprecipitated it is almost impossible to extract appreciable activity from the precipitate. To lessen this coprecipitation, the bulk of the extraneous protein was precipitated with 45 per cent acetone and then more was removed with 50 per cent acetone.

The precipitate obtained by treatment with 55 per cent acetone contained much insoluble material, and the uricase had to be extracted from the precipitate. This extraction proceeded slowly and many washings were required to remove most of the enzyme. After each washing the insoluble material showed less tendency to coagulate, so that the last few extractions were made with water, which does not give as fine a suspension as buffer, and the insoluble material was removed by centrifugation for one hour at 20,000 x g. Perhaps this extraction would proceed better if a more alkaline buffer were used. In small scale trial experiments these steps generally resulted in 20-30 fold purification. When this procedure was applied on a large scale it appeared to be less efficient but the exact purification was not determined.

Wholly satisfactory results were never obtained with $(NH_4)_2SO_4$ fractionations and even under the mildest conditions about 30 per cent of the activity was lost, but in small scale experiments sufficient purification was obtained to make the fractionation worth-while. In these trial experiments uricase was precipitated between 50 and 65 per cent saturation with $(NH_4)_2SO_4$, at pH 7 or slightly above. In the actual large scale fractionation as shown in Figure 6, most of the activity came down at 50 per cent saturation with $(NH_4)_2SO_4$ (pH 7), and only a very small amount was found in the 50 to 65 per cent saturation fraction. A possible reason for this change in behavior is that the extract from the acetone precipitate contained an appreciable amount of acetone which had evaporated from the sample used to work out the ammonium sulfate fractionation while it stood in the cold room several days, but was retained in the bulk of the material which was stored tightly stoppered in the deep freeze. Another possible reason for this behavior is that the addition of boiled extract caused sufficient lowering of pH and increase of salt concentration to affect the fractionation. Boiled extract was added to the material being fractionated as a possible preventive to the loss of activity occurring during ammonium sulfate fractionation, since the activating effect of boiled extract had just been discovered and was thought to be due to replacement of a cofactor which was removed by ammonium sulfate treatment. However, it had no apparent effect on preservation of activity, and since later experiments give little evidence that a cofactor is involved in the activation, this treatment could probably be omitted.

In order to obtain good results with the acid precipitation technique, great care must be used in adjusting the pH to the values given in

Figure 6. Adjustment to pH 4.5 without the intermediate pH 5.0 results in precipitation of a large amount of enzyme, and allowing the pH to get as low as 4.0 causes destruction of most of the activity. In trial experiments, when 0.1 N HCl was used for acidification, almost quantitative yields of enzyme were obtained. In the large scale preparation, 1 N HCl was used for acidification since much more material was being handled, and a considerable amount of the activity was lost. This loss of activity might well be due to the presence of localized regions of low pH during the addition of the stronger acid, and could probably be avoided by slower addition of acid and better stirring.

At this stage of purification the enzyme is very unstable in solution in the presence of fractionating agents which previously had little effect on the enzyme. For example, 50 per cent of the activity is destroyed when the enzyme is allowed to stand for three hours at pH 7 in the presence of 55 per cent acetone and 60 per cent or more is destroyed on standing for a similar time at pH 7 in the presence of 50 per cent saturated $(NH_4)_2SO_4$. The destruction of the enzyme by $(NH_4)_2SO_4$ is somewhat reduced by making the solution 0.01 M in sequestrene (ethylenediaminetetraacetic acid) but 0.001 M sequestrene has no effect on the instability toward acetone. However, it was found that if sufficient acetone was added to precipitate the uricase immediately, little activity was lost. Therefore, the enzyme was precipitated in this way and, after removal of acetone from the precipitate by storage in a vacuum desiccator over a solution of 2,4-dinitrophenylhydrazine in dilute sulfuric acid, the enzyme was easily extracted by washing the precipitate twice with small volumes of distilled water leaving behind a fairly large insoluble residue.

Finally the enzyme solution was dialyzed against 0.04 M tris-hydroxy-methyl-amino-methane buffer, pH 8.2 (at 10°C.), which was 0.001 M in sequestrene and fractionated by electrophoresis on Professor Mitchell's sponge electrophoresis apparatus for five to six hours with a potential of 500 volts. This apparatus consists of a lucite trough, 50 cm. long, 2 cm. wide, and 2 cm. deep, which is suspended between two lucite electrode vessels. For use the trough is packed with small blocks of foam rubber, 12 mm. high, 20 mm. wide and 7-12 mm. thick, which are saturated with buffer. The material to be fractionated is injected between two of these blocks with a syringe, and potential is applied across the electrode vessels for the desired length of time. The fractions are recovered by removing the sponges from the trough individually and expressing their contents with a 10 ml. syringe. The active fractions from the electrophoresis apparatus are the purest enzymes yet obtained, having specific activities ranging from two to four and one-half times greater than that of the product of the previous step.

Over-all Purification. The extent of uricase purification obtained by use of the above fractionation procedure can be calculated by comparison of the "apparent turnover number" of uricase in the crude extract with that in the purest fraction. For the purpose of calculating these "apparent turnover numbers" Neurospora uricase is assumed to have a molecular weight of 10^5 and a nitrogen content of 16 per cent. Activity was determined as described in Section III-C; incubation mixtures had a volume of five ml. and contained one mg. of uric acid.

	Crude extract	Electrophoresis fraction
pH of incubation mixture	8.5	8.8
optical density decrease in 20 min.	0.99	0.46

	Crude extract	Electrophoresis fraction
mg. of uric acid destroyed in 20 min.*	0.707	0.329
μM of uric acid destroyed in 20 min.	4.21	1.96
μM of uric acid destroyed per min.	0.201	0.0977
γ of protein nitrogen	720.	0.717
γ of protein	4500.	4.48
μM of protein	0.0450	4.48×10^{-5}
"apparent turnover number" ($min.^{-1}$)	4.67	2.18×10^{3}
"apparent turnover number"** (corrected to pH 8.8) ($min.^{-1}$)	5.08	2.18×10^{3}

By calculating the ratio of these "apparent turnover numbers" it is seen that an over-all purification of 430 fold has been obtained by the use of this fractionation method.

Additional fractionation experiments. Several other fractionation techniques were tested but not used for various reasons. The results of some of these attempted fractionations are given below for reference in further purification work.

In an attempt to remove extraneous protein, aliquots of crude Neurospora extract were heated in a water bath at 55°C. for various periods of time. Some destruction of uricase had occurred after two minutes and all the activity was gone after four minutes of heating in this fashion. It is possible that this rapid destruction of activity is due to the presence of proteases and that this technique might work on purer preparations,

---

* Optical density decrease / 1.40.

** The rate at pH 8.5 is approximately 92 per cent of that at pH 8.8.

but an unheated control of this extract seemed to be more stable than usual to storage in the refrigerator, indicating a low rate of proteolytic destruction of uricase.

The manganous hydroxide-manganous phosphate gel precipitation used by Professor Horowitz (50) to remove any insoluble material and some protein from Neurospora extracts before tyrosinase purification was tested. This procedure consists of making the extracts 0.05 M in K_2HPO_4 and adjusting the pH to 7 followed by the addition of 0.05 volume of 1 M $MnSO_4$ and readjustment of the solution to pH 7. The flocculent precipitate which forms is removed by centrifugation leaving a clear supernatant. About 30 to 40 per cent of the uricase was precipitated by this treatment and it could not be removed by washing with water. The specific activity of uricase in the supernatant is decreased by this procedure.

The stability of uricase to several solvents was tested by adding one volume of a given solvent to an aliquot of Neurospora extract and allowing the mixture to stand in the refrigerator for four days. At the end of this period the solvents were removed by lyophilization against liquid nitrogen and the dry samples were suspended in water and assayed. When tested in this way it was found that pyridine, n-propanol and methylethyl ketone destroyed all the activity, and that dioxane and t-butanol destroyed most of it, while methanol, ethanol and iso-propanol gave results similar to those obtained with acetone, and would supposedly be useful as fractionating agents. However, when fractionation with methanol was attempted most of the uricase was destroyed. In addition, when the stability of uricase toward methanol was retested by the above method, all of the activity was destroyed, even though in the same experiment the enzyme was stable to acetone. The reason for this discrepancy is not known.

Acetone fractionation was tested at pH 8.5 and pH 10.0 but the enzyme was precipitated by approximately the same acetone concentrations as it was at pH 7 and a greater amount of activity was destroyed at the higher pH values.

After electrophoresis in tris-hydroxymethyl-amino-methane buffer as described above, the active fractions were combined, concentrated by lyophilization, and dialyzed against 0.04 M cacodyllate buffer, pH 6.3 (10° C.). After running this material on the electrophoresis apparatus, it was found that only a trace of activity remained, although the enzyme is not inhibited by cacodyllate, and at this stage of purification is not greatly affected by lyophilization or dialysis. The activity was not recovered by recombination of all the electrophoresis samples nor by incubation with boiled extract. The problem of uricase destruction by mild treatments such as the above is discussed in Section IV-B.

E. Preparation of Boiled Extract

The boiled extract used in this study was prepared either by heating ground whole Neurospora suspended in one volume of water (or a centrifuged extract) in a boiling water bath for eight to ten minutes, or by boiling two minutes or longer with one volume of ethanol. The resulting mixture was centrifuged and the precipitated material washed and discarded. The combined supernatant and washings were used directly as the source of activator in all subsequent enzyme experiments (after removal of ethanol when it was used). Heating for shorter periods in aqueous solution is unsatisfactory because the activating substance is unstable in the resulting solution and it disappears on storage in the refrigerator for two or three days. This destruction appears to be enzymatic in nature since it was observed that at pH 8, incubating for two hours at

35°C. or heating in a boiling water bath for a short time destroyed the activity, while heating in a similar manner at pH 6 or pH 13 had little effect.

Some fractionation experiments were done in an attempt to isolate and identify the activating substance or substances. Extraneous material was precipitated by adjusting the boiled extract to pH 9 with $Ba(OH)_2$, removing the precipitated material, and readjusting the pH to 7 with H_2SO_4 to remove the excess barium. Judging from the size of the precipitate a fairly large amount of material was removed, but almost all the activity remained in the supernatant. When the boiled extract was treated with three times its dry weight of Norit A, the active factor was adsorbed. Extraction of the charcoal with boiling ethanol eluted only about one-third the activity. No activity was recovered when the charcoal was eluted with 5.0 per cent pyridine, 5.0 per cent aniline, 0.1 per cent NH_3, or 0.1 per cent NH_3 in 60 per cent ethanol. Precipitations with lead, silver, and mercury were attempted, but during the course of these experiments, it was found that sulfide, which was used to remove the excess heavy metal ions, has an effect on the enzyme which masks that of the activating substance. In some cases silver and mercury formed colloidal sulfides which could not be removed. Heating these fractions in acid solution would remove the excess sulfide and probably would coagulate the colloidal precipitates, but unfortunately the activating substance is highly acid unstable so this treatment cannot be used. Further fractionation was not attempted because of shortage of time and because the importance of the activating substance to the reaction was not clear. The nature of this activation is discussed in Section IV-B.

F. Determination of Protein Nitrogen

Protein nitrogen was determined by a micro-Kjeldahl-Nesslerization method. In the technique generally used during the course of these investigations, color was developed with 5 ml. of Nessler's Reagent (Eimer and Amend) in a total volume of 35 ml., and best results were obtained in the range of 10-100γ of protein nitrogen. A modification of this method for determining 1-15γ of protein nitrogen was made by developing the color with one ml. of Nessler's Reagent in a total volume of 7 ml. In the micro-modification it is necessary to neutralize the digestion mixture before addition of Nessler's Reagent.

G. Chromatographic Techniques

With the objective of identifying the products and possibly some of the intermediates of uric acid oxidation, preliminary experiments by paper chromatography were done in order to find a system which would be useful for separating these compounds. The Rf values of uric acid, allantoin and uroxanic acid in various solvent mixtures are given in Table IV.

The product from permanganate oxidation of uric acid, according to the method of Schuler and Reindel (41) for preparation of hydroxyacetylene-diureine-carboxylic acid, was chromatographed with the lutidine-water mixture. The Rf of this compound is the same as that of allantoin, which may mean that it is converted to allantoin in the presence of lutidine. The streaking of uroxanic acid may be due to formation of complexes with heavy metal ions and better results might be obtained with the use of washed paper.

Color reagents. The sprays used for detection of the above compounds were described in the University of Texas bulletin on paper

Table IV

Rf VALUES OF URIC ACID ALLANTOIN AND UROXANIC ACID IN VARIOUS SOLVENTS

Solvent	Rf		
	Uric acid	Allantoin	Uroxanic acid
n-propanol-0.6N NH_3 (5:1)	0.04	0.08	--
dimethyl formamide	0.11	0.96	--
dimethyl formamide-conc. NH_3 (100:1)	0.07	0.85	--
dimethyl formamide-pyridine (1:1)	0.08	0.81	--
2,6-lutidine-water (65:35) (51)	0.39	0.58	0.19*
ethanol-1 N acetic acid (3:1)	0.28*	0.42	0.23*
ethanol-1 N NH_3 (3:1)	0.18*	0.30	0.04*

* Streaked from origin.

chromatographic methods (51). Uric acid was detected by spraying the paper with a 1:1 mixture of 0.1 N $AgNO_3$ and 5 N NH_3, which develops a black spot on a white background. A two per cent solution of p-dimethyl-amino-benzaldehyde in 1.2 N HCl was used for detection of the other compounds. This reagent gives a yellow color with allantoin, urea and related compounds.

H. Chemical Preparations

Synthesis of uroxanic acid. Uroxanic acid (D - fig. 1) was prepared according to the method of Behrend and Schultz (52). Uric acid (11.2 grams) was dissolved in 225 ml. of water containing 20 g. KOH, and 140 ml. of 5 per cent $KMnO_4$ were added in small portions at room temperature. When the oxidation was complete the MnO_2 was removed by vacuum filtration. The filtrate was concentrated to 40 ml. under reduced pressure and stored in the refrigerator overnight. The crystalline product was recovered by filtration and recrystallized from 10 ml. of boiling water. The product was dried in a desiccator over P_2O_5. Yield of dipotassium salt 2.0 g. Theory 19.7 g. The free acid was prepared by saturating a solution with potassium uroxanate at 0°C. and adding concentrated HCl to the cold solution until the pH was less than 2 as determined by universal indicator paper. The precipitated acid was recovered, washed with a small volume of cold water and dried in a desiccator over P_2O_5. The acid was not recrystallized because of its insolubility in cold water and instability in solution at higher temperatures.

Analyses of Product - Calculated for $C_5N_4H_8O_6$: C - 27.30%, H - 3.67%, N - 25.4%, neutralization equivalent, 110. Found: C - 27.42%, H - 4.22%, N (Dumas) - 23.68%, N (Kjeldahl) - 24.32%, neutralization equivalent, 111. Absorption spectrum: A 70γ/ml. solution of dipotassium

uroxanate has negligible optical density in the wavelength band from 225 mμ to 350 mμ.

Preparation of hydroxyacetylene-diureine-carboxylic acid. Uric acid was oxidized with alkaline permanganate by a modification of the method of Schuler and Reindel (41) for preparation of the trisilver salt of hydroxyacetylene-diureine-carboxylic acid. Uric acid (0.11 gram) was suspended in 9 ml. cold water and dissolved by the addition of 1.05 ml. 20 per cent KOH. The solution was placed in an ice bath and the temperature maintained between 0 to 5°C. while 2.6 ml. of 5 per cent $KMnO_4$ was added in small portions. When the oxidation was completed, the MnO_2 was removed by centrifugation and the product precipitated by the addition of 1 g. $AgNO_3$ dissolved in 1 ml. water. The precipitate was washed three times with water, twice with absolute ethanol, once with ether, and stored over P_2O_5 in a vacuum desiccator in the refrigerator. In an attempt to recover a soluble salt of the product, the silver precipitate was suspended in 15 ml. of 0.3 N NH_3 and decomposed with H_2S. The AgS was removed, washed, and the supernatant solutions were combined and evaporated to 4 ml. while frozen. After addition of ethanol and ether to this solution a fine white precipitate formed which was recovered, redissolved and reprecipitated. Examination of this precipitate under a binocular microscope showed that it consisted of small white needle-like crystals. Only a few milligrams of this product were obtained so it was not properly characterized. However, when this product was chromatographed with lutidine-water, a single p-dimethyl-aminobenzaldehyde-reacting spot was obtained with the same Rf as that of allantoin. Its absorption spectrum (fig. 7) differs from those of uric acid (fig. 8), allantoin (fig. 9) and uroxanic acid.

Further attempts to prepare hydroxyacetylene-diureine-carboxylic acid were made with the use of the catalytic oxidation method of Schuler and Reindel (42). Various methods for isolation of the product without use of silver precipitation were tested, but the only spectrally detectable compound obtained was allantoin. It should be pointed out that in the work of Schuler and Reindel, hydroxyacetylene-diureine-carboxylic acid was precipitated as the silver salt and was not further purified. Although this product gave reasonably good analytical values its composition is somewhat questionable, since it has such a high percentage of silver that small deviations in the carbon, hydrogen and nitrogen values might be obscured.

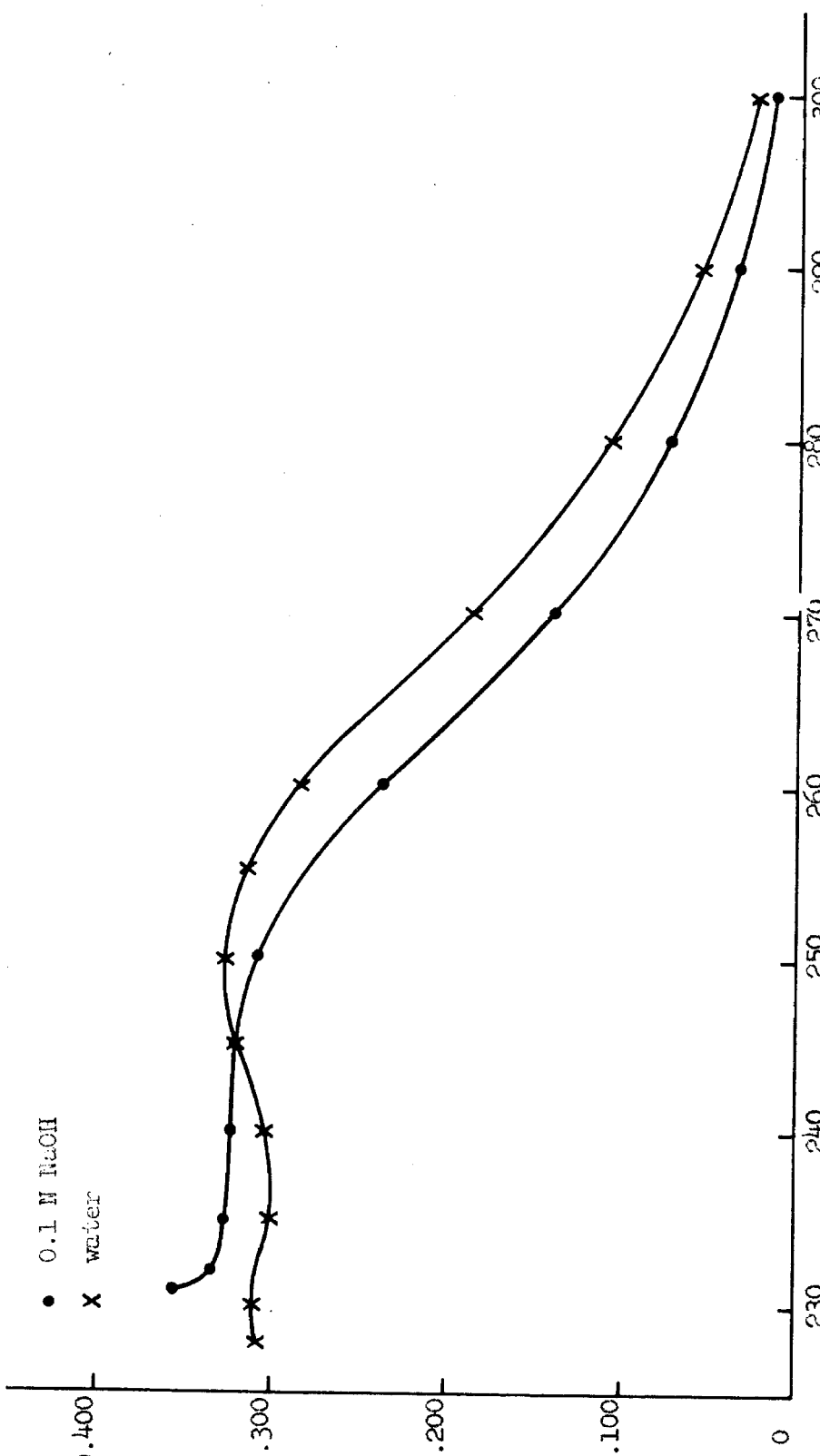


Figure 7 ABSORPTION SPECTRUM OF THE PRODUCT FROM PERMANGANATE OXIDATION OF URIC ACID (20 γ/ml.)

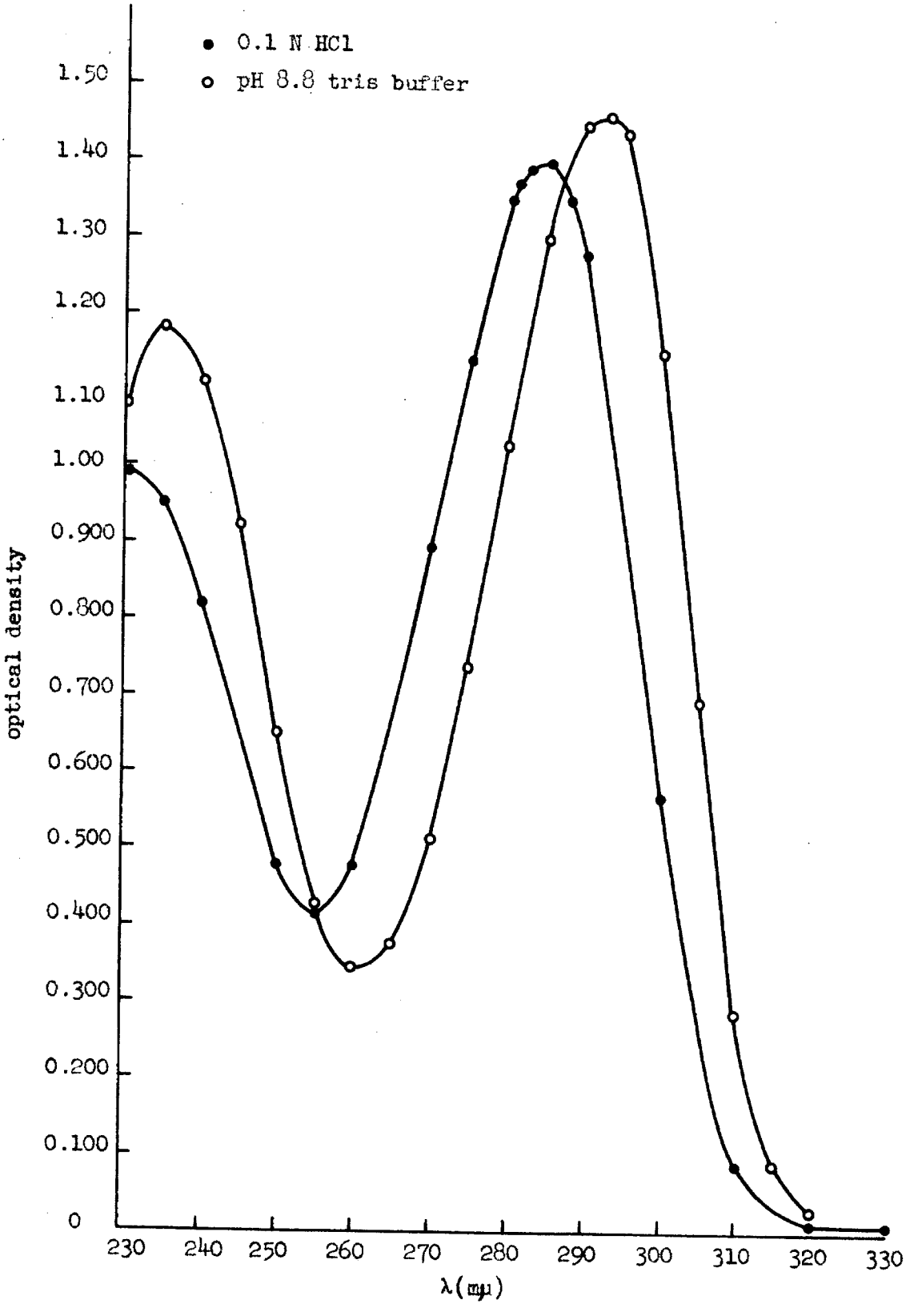


Figure 8 ABSORPTION SPECTRUM OF URIC ACID (20 γ/ml.)

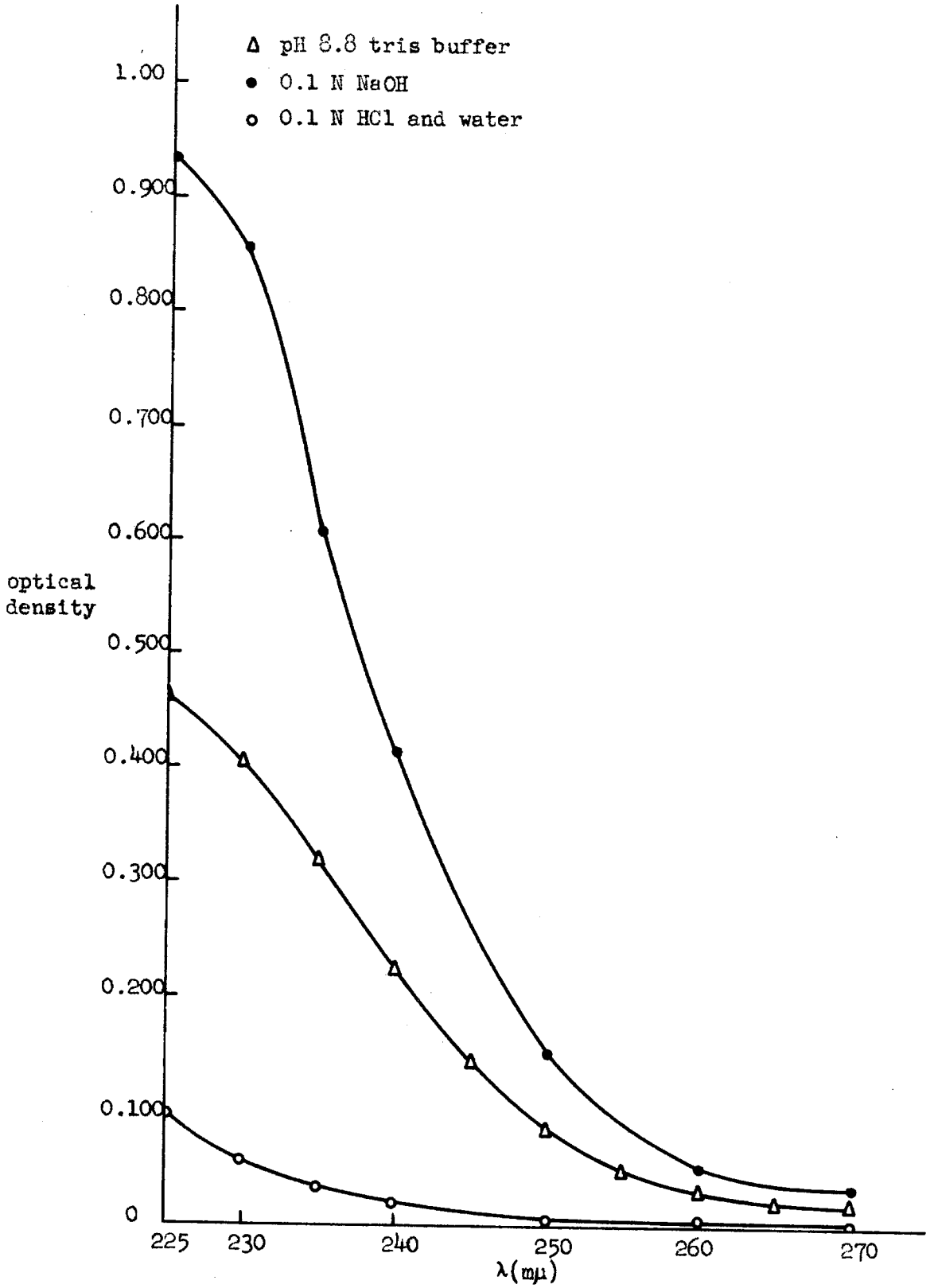


Figure 9 ABSORPTION SPECTRUM OF ALLANTOIN (20 γ/ml.)

IV. RESULTS

A. The Effects of Supplementing the Growth Medium with Uric Acid

Adaptive formation of uricase. The effects of uric acid addition to the growth medium were investigated in an attempt to find means for increasing the uricase concentration of Neurospora crassa 5297a. It was found that some adaptation does occur and that the specific activity of uricase in crude extracts of mold grown on Fries minimal medium 2×10^{-3}M in uric acid is about two times greater than that obtained in extracts of mold grown on unsupplemented medium. "Apparent turnover numbers" for the two types of extracts can be calculated by the same method used in Section III-D.

	Unsupplemented	2×10^{-3}M uric acid
optical density decrease in 20 min.*	0.31	0.99
mg. uric acid destroyed in 20 min.	0.221	0.707
µM uric acid destroyed in 20 min.	1.32	4.21
µM uric acid destroyed per min.	0.0659	0.210
mg. protein nitrogen	0.44	0.72
mg. protein**	2.75	4.50
µM protein**	0.0275	0.0450
"apparent turnover number" ($min.^{-1}$)	2.40	4.67

ratio $4.67/2.40 = 1.95$

---

* Both extracts assayed at pH 8.5.

** Assuming a nitrogen content of 16 per cent and a molecular weight of 10^5.

Growth stimulation by uric acid. During the course of the above investigation, it was observed that addition of uric acid to Fries minimal medium has a stimulating effect on the growth of 5297a, as is shown in Table V.

Table V

THE EFFECT OF URIC ACID ON GROWTH OF 5297a*

Growth period (hrs.)	Dry weight (mg.) (average of two flasks)	
	Unsupplemented	2×10^{-3}M uric acid
24	18.8	36.2
28	52.9	82.7
32	92.8	124.6
38	145.4	153.9
50	130.9	131.4

* Incubated at 25°C. with shaking.

The mechanism of this stimulation is not known but probably it is due to utilization by the mold of the breakdown products of uric acid, as precursors of essential compounds.

B. The Properties of Neurospora Uricase

Purity of the enzyme. The relative purity of the uricase obtained by use of the fractionation procedure devised in this study can be calculated by comparison of its specific activity with those of enzymes obtained by other workers. Such a comparison is made below with the enzyme prepared by Holmberg (21), since the specific activity reported for his

enzyme is higher than those of the preparations made by other workers. Specific activity of Holmberg's enzyme:

Q_{O_2}	6000 µL O_2/hr./mg.
nitrogen content	13.5%
oxygen uptake	741 µL O_2/min./mg. prot. N

Specific activity of the purest enzyme obtained in this study:

uric acid destruction	0.0977 µM/min.
protein nitrogen	0.717γ
uric acid destruction	136 µM/min./mg. prot. N
oxygen uptake	1525 µL O_2/min./mg. prot. N

Ratio of specific activity of the enzyme prepared in this study to that of Holmberg's enzyme: 2.06.

Thus, the uricase prepared by this method is approximately two times more active than the purest enzyme previously obtained.

pH optimum. Early experiments with crude enzyme preparations showed that Neurospora uricase was active in the range from pH 6 to pH 11, and that the point of optimal activity was between pH 8 and pH 9. Later, more refined, experiments using a purified enzyme showed that the maximal rate was obtained at pH 8.8. These results are summarized in Figure 10.

Nature of the active groups of the enzyme. In order to obtain information about the active groups of the enzyme, several experiments have been done regarding the effects of various additives on the reaction rate. The effects of some commonly used inhibitors are shown in Table VI. The results of these inhibition studies are very similar to those obtained by other workers using enzymes from animal sources (21, 22, 29), and it appears that the enzymes are functionally similar in spite of

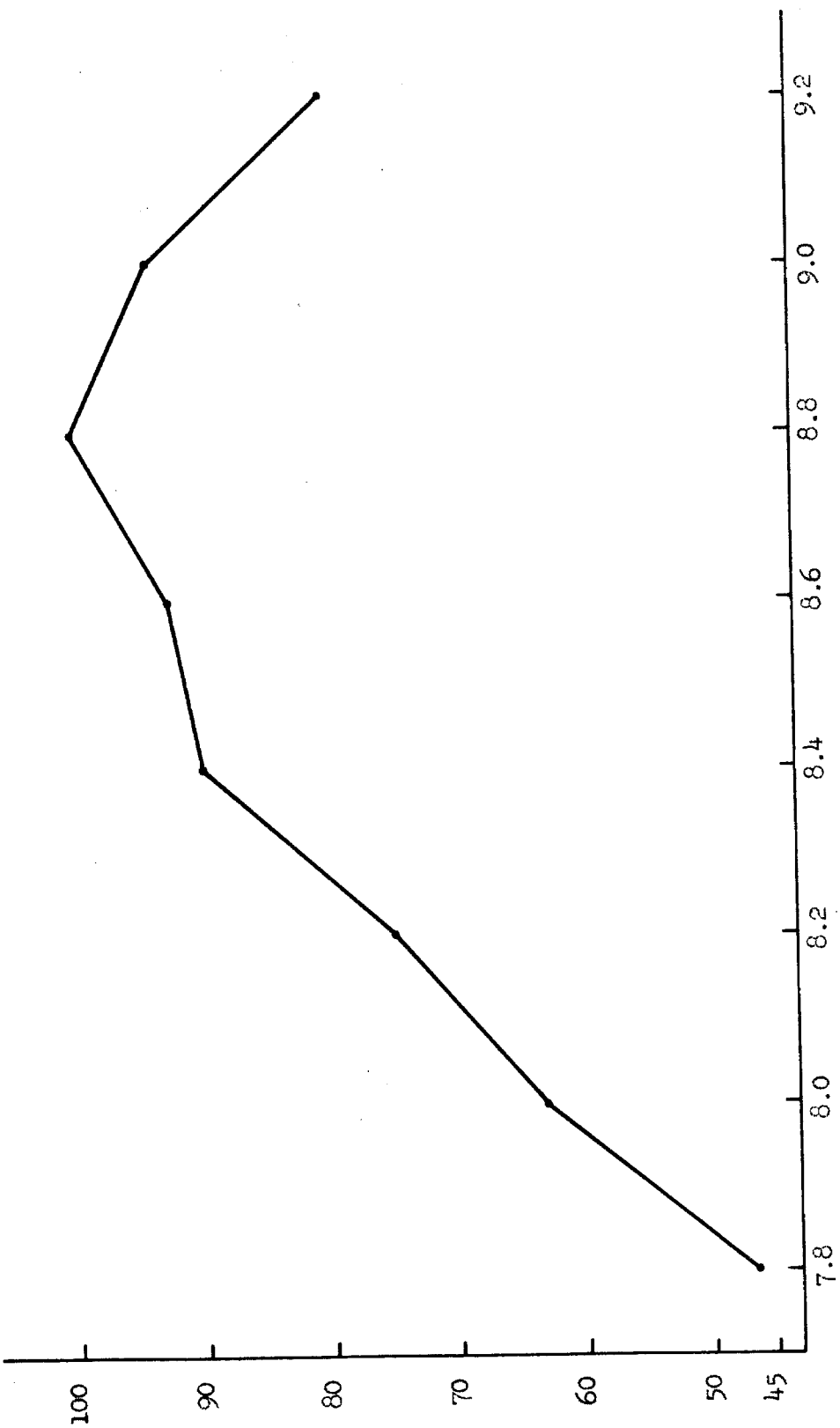


Figure 10 pH OPTIMUM OF URICASE FROM NEUROSPORA CRASSA

Table VI

EFFECTS OF INHIBITORS ON NEUROSPORA URICASE

Inhibitor	Molar concentration	% Inhibition
KCN	0.001	100
NaN_3	0.01	26
NaF	0.1	12
Na_2HAsO_4	0.001	8
$NH_2OH \cdot HCl$	0.02	77
sequestrene	0.001	0
p-chloromercuribenzoate	0.0004	21

differences in intracellular localization and physical properties. Again the most clear-cut result is the inhibition by cyanide which would indicate participation of a heavy metal ion. However, the result with sequestrene indicates that if such an ion is involved, it is probably very strongly bound to the enzyme. The effect of p-chloromercuribenzoate on the reaction rate indicates that if sulfhydryl groups are required for uricase activity, the more essential groups are protected in some way from this blocking agent. Information as to the nature of this partial inhibition can be obtained by closer observation of the time course of the inhibited and uninhibited reactions.

Additive	Optical density decrease during designated interval (min.)		
	0-20	20-40	40-60
none	0.26	0.18	0.16
p-chloromercuri-benzoate	0.16	0.15	0.15

From these data it can be seen that the initial inhibition by p-chloromercuribenzoate is high, but that the rate of the inhibited reaction remains constant, while that of the uninhibited reaction decreases. As has been previously mentioned, addition of glutathione to the reaction mixture also prevents this decrease in rate (fig. 11). These two observations indicate that sulfhydryl groups are involved in the reaction. If this indication is correct, the observed rate decreases during the course of the reaction could be explained by oxidation of these groups and the glutathione activation by their reduction. If such is the case, it would appear that any sulfhydryl groups not affected by p-chloromercuribenzoate are also protected from oxidation.

Uricase is activated by fairly strong reducing agents, such as glutathione (fig. 11) and sulfide (fig. 12), and by some unknown compound or compounds present in a boiled extract of Neurospora (fig. 12). The mechanism of sulfide activation is probably the same as that of glutathione activation, and the initial inhibition shown in the sulfide curves is probably due to limitation of oxygen caused by a relatively high concentration of reducing agent. However, for several reasons it is believed that the activating substance in boiled extract acts by a different mechanism. First, as can be seen in Figure 12, after the initial inhibition is overcome the reaction mixture containing both boiled extract and sulfide appears to have a higher activity than that containing only sulfide, although sulfide is present in excess in both reaction mixtures. Second, it is possible to obtain an enzyme which is stimulated by glutathione and unaffected by boiled extract (the material obtained after pH fractionation - fig. 6).

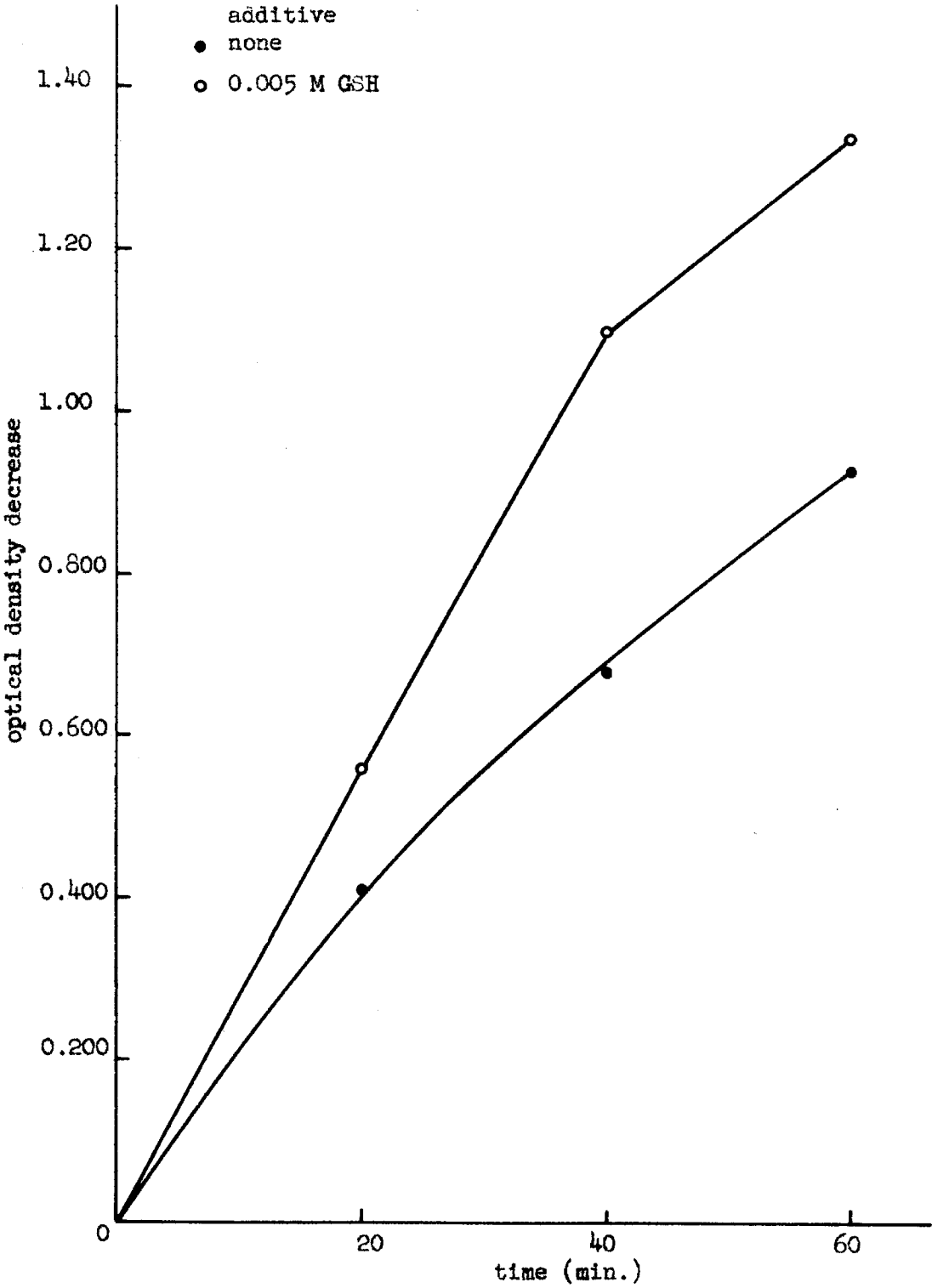


Figure 11 EFFECT OF GLUTATHIONE ON URICASE

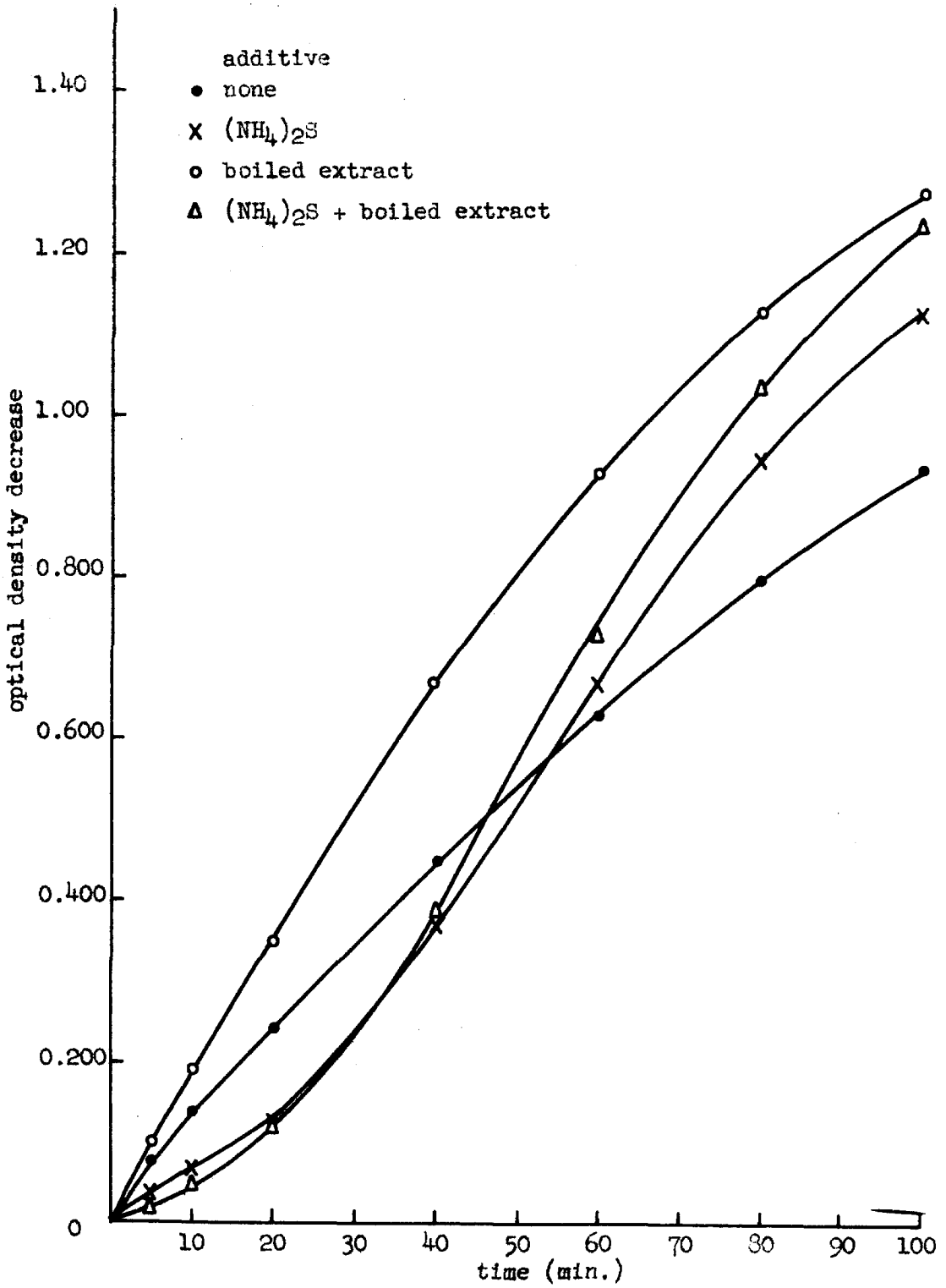


Figure 12 EFFECT OF $(NH_4)_2S$ AND BOILED EXTRACT ON URICASE

Additive	Optical density decrease	
	20 min.	40 min.
none	0.35	0.64
boiled extract	0.36	0.68
GSH	0.51	1.07

Finally the activating substance in boiled extract is destroyed by boiling for 20 minutes in 0.1 N acid but survives this heating in 0.1 N base (fig. 13). This behavior would not be expected of most biological reducing agents which are destroyed more rapidly in base than in acid. A further observation of the nature of the activating substance was made by incubation of boiled extract with intestinal phosphatase for two hours at pH 8 and 35°C. This treatment had no effect on the activation produced by the preparation.

A number of attempts were made to deactivate uricase by mild techniques in order to find if the boiled extract contained a dissociable cofactor required for uricase activity. Procedures tested were dialysis against saturated $(NH_4)_2SO_4$ at pH 5 and pH 7, electrodialysis at pH 4.5, pH 6 and pH 7, and aging in dilute solution at pH 6.5. Some restoration of activity was obtained by addition of boiled extract to aged and electrodialyzed preparations, but the activation never exceeded two fold. In the cases where all or most of the uricase activity was destroyed, no activation was observed.

A further attempt to discover a cofactor was made by taking the absorption spectrum of purified uricase (fig. 14). The absorption spectrum of the enzyme was that of a simple protein (53), and none of the absorption peaks characteristic of the heterocyclic ring-containing cofactors were visible. However, this does not prove that these compounds

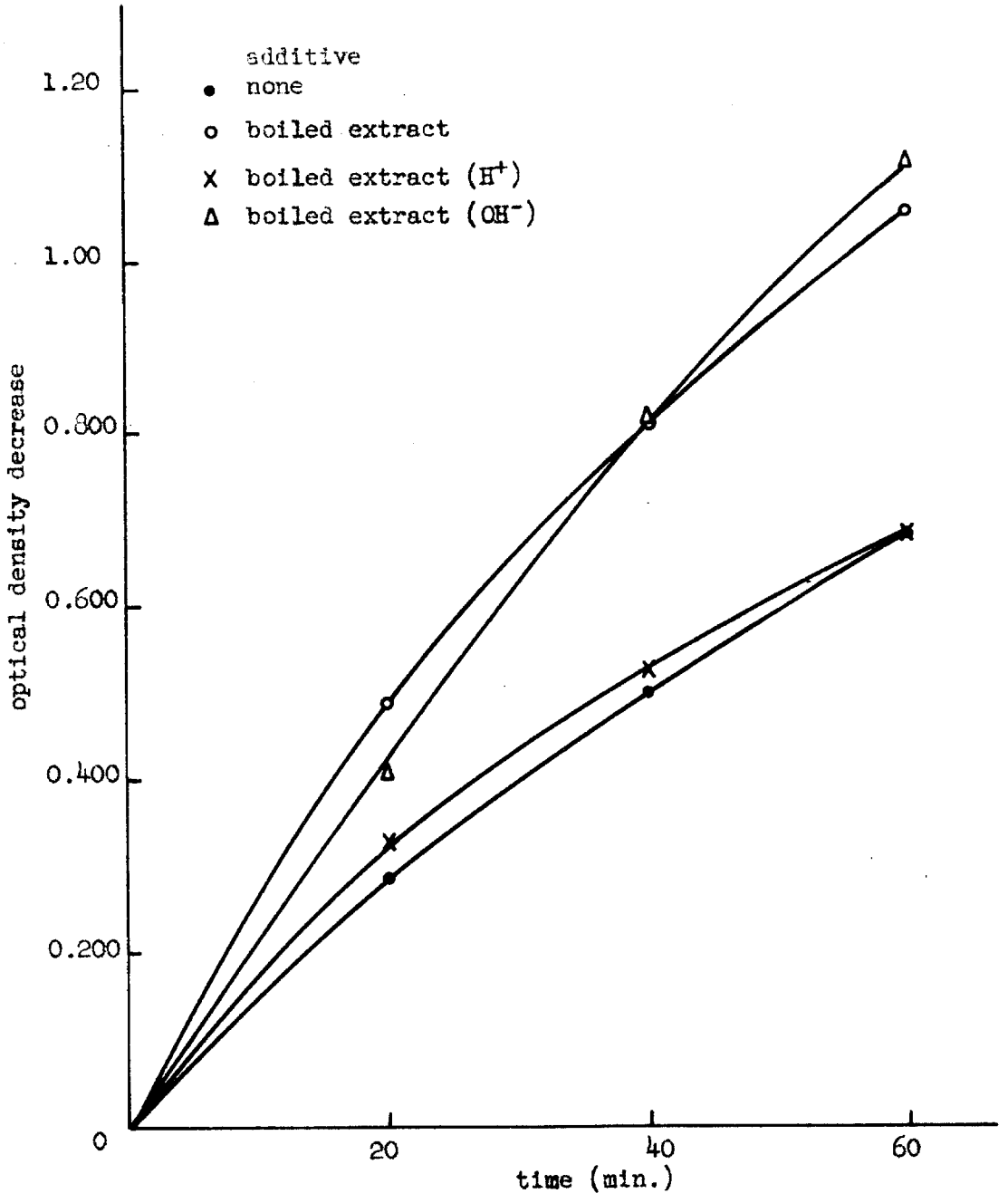


Figure 13 EFFECT OF ACID AND BASE TREATMENT ON BOILED EXTRACT

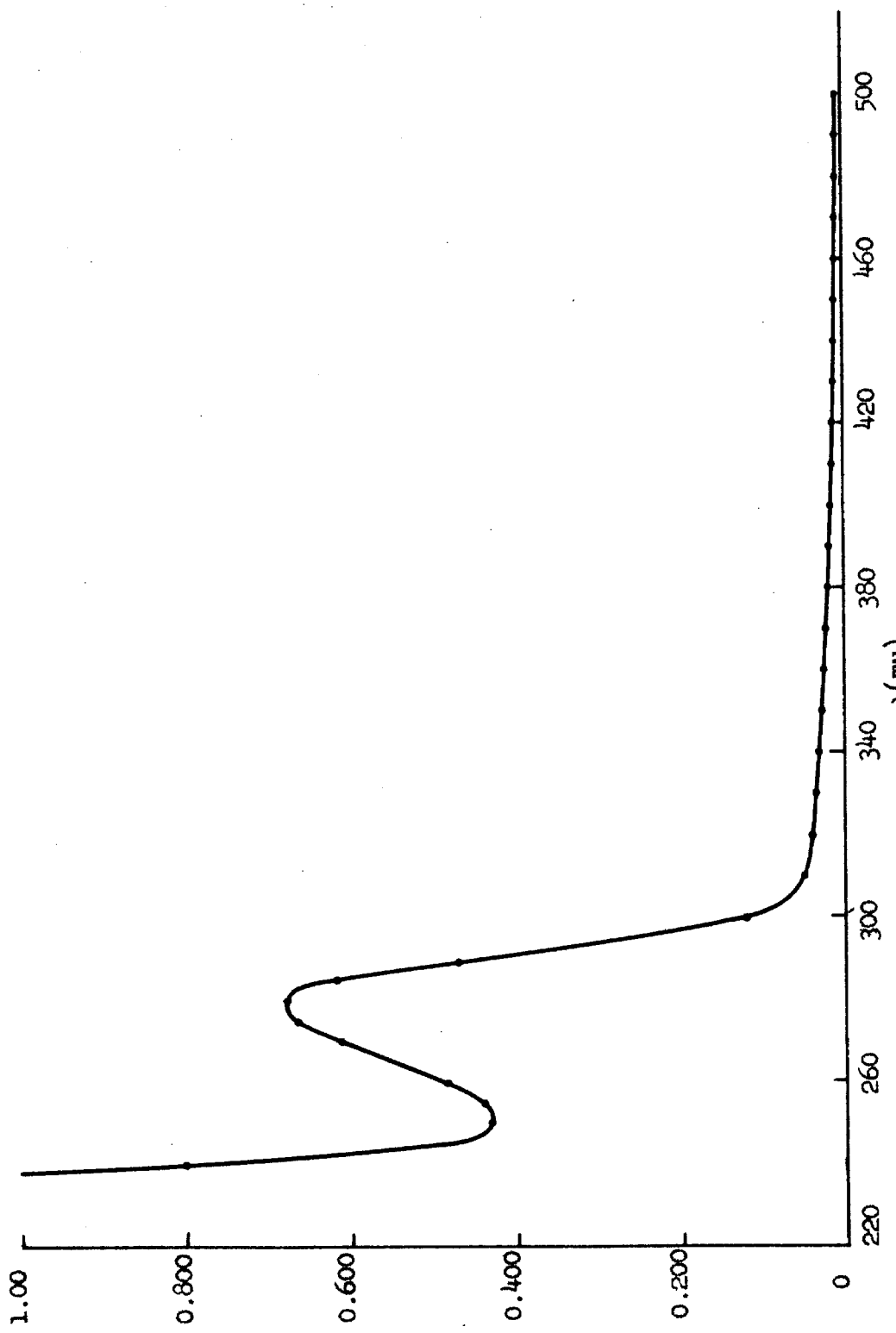


Figure 14 ABSORPTION SPECTRUM OF PURIFIED URICASE (71.7 γ N/ml.)

are not present; if the molecular weight of the enzyme is assumed to be 10^5 it can be calculated that the increase in optical density caused by one molecule of adenine per molecule of enzyme would be of the order of 0.06 if the enzyme were pure. If this is the case, it would be difficult to detect these compounds by direct observation of the enzyme if the purity was less than 30 per cent.

Although there is no direct evidence that uricase requires a co-factor, it seems likely that the destruction of activity by treatments such as dialysis against $(NH_4)_2SO_4$ and electrophoresis at pH 6.3 would be due to a dissociation. Since uricase is an oxidase, it would be especially interesting to know what the active groups are if one of the known dissociable cofactors is not involved. Further observations of the properties and stability of an enzyme of known high purity will probably give solutions to these problems.

C. Experiments on the Nature of the Uric Acid Oxidation

Oxygen uptake. The stoichiometry of oxygen uptake was determined by carrying out the reaction with addition of catalase in Warburg Respirometers until about 80 per cent of the stoichiometric amount of oxygen had been taken up. The reaction was then stopped by the addition of sulfuric acid and the remaining uric acid was determined spectrophotometrically. The results of duplicate determinations are shown below.

Incubation temperature - 37°C.

Initial uric acid concentration - 600 γ/ml.(1.8 mg. in 3 ml.)

oxygen uptake (found)	1. 109 µL	2. 104 µL
decrease in optical density (1:30 dilution)	1. 1.11	2. 1.09
uric acid destruction	1. 1.43 mg.	2. 1.34 mg.

oxygen uptake (calculated)	1. 95.3 µL	2. 89.6 µL
Ratio (oxygen uptake) (found/calculated)	1. 1.15	2. 1.16

Thus, it is seen that in the presence of catalase approximately one atom of oxygen is taken up per mole of uric acid oxidized.

Spectrophotometric analysis of the uric acid oxidation. As mentioned above, Praetorius (46) obtained spectrophotometric evidence for the existence of at least two transitory intermediates during the course of uric acid oxidation catalyzed by animal uricase. In order to determine whether these substances are produced by the enzyme from Neurospora, absorption spectra were taken of a reaction mixture after incubation of the enzyme with uric acid for varying periods of time. High levels of uricase were used in the reaction mixtures for this experiment so that any spontaneously decaying intermediate would pile up. Incubation with shaking at 35°C. was carried out with a uric acid concentration of 200γ/ml. After incubation for a given interval an aliquot was taken, diluted ten fold with cold water and optical density readings were made at ten mµ intervals from 240 mµ to 340 mµ as rapidly as possible. In all cases these readings were completed within 3 to 3.5 minutes after the sample was taken. For the purpose of determining the uric acid concentrations a second aliquot was taken at the end of the incubation period and diluted ten fold with 0.002 M KCN. This procedure was used because Praetorius had reported that cyanide stopped the uricase oxidation but did not interfere with the decay of the intermediates. Spectra were taken of these samples after they had been allowed to stand at room temperature for several hours. Unfortunately the intermediates did not completely decay and the cyanide samples had greater optical density at some wavelengths

than could be accounted for by uric acid and allantoin. These data are given in Figures 15-26. Three curves are given for each time interval; they are the absorption spectrum of an aliquot of the reaction mixture, the difference curve obtained by subtraction of the absorption spectrum of the cyanide treated sample from that of the untreated sample and last, assuming the absorption at 290 mμ of the cyanide treated sample to be entirely due to uric acid, the difference curve obtained by subtraction of the calculated uric acid absorption spectrum from that of the cyanide treated sample. The total absorption due to allantoin, if the maximum calculated amount were present, is also plotted on the difference curves. This analysis was carried out for incubation periods of two, five, ten and fifteen minutes.

It can be seen from these data that there are at least two transitory intermediates as Praetorius reported for animal uricase and possibly the absorption in the shorter wavelength region is due to more than one compound.

Since the intermediate absorbing in the longer wavelength region has a low optical density and appears only in the two minute sample, an additional experiment was performed to establish its existence. In this experiment optical densities were read directly from the reaction mixture rather than from a diluted aliquot. Changes in absorption at wavelengths longer than 320 mμ were recorded. From time to time the reaction mixture was removed from the spectrophotometer and aerated by shaking. In addition to establishing the existence of this intermediate, these data show that it is very unstable and that its rate of production responds very rapidly to conditions which should alter the rate of uric acid oxidation.

Time	λ(mμ)	Optical density	Time	λ(mμ)	Optical density
0	320	0.211	3'20"	340	0.030
0	325	0.053	5'5"	320	0.205
0	330	0.023	20'30"	320	0.180
0	335	0.018	*22'15"	320	0.280
0	340	0.016	24'50"	320	0.217
1'20"	320	0.561	*26'50"	320	0.315
2'0"	325	0.217	34'30"	320	0.140
2'50"	330	0.088	*37'20"	320	0.235

Although absorption curves obtained in these experiments are almost certainly not due to any single compound it should be pointed out that the absorption spectrum of the permanganate oxidation product (Section III-H) is such that this compound may actually be one of the intermediates contributing to absorption at the shorter wavelengths. Alteration of the experimental procedure so that uric acid concentration could be accurately determined would give curves more characteristic of the intermediate compounds. In addition it may be possible to isolate the compound or compounds absorbing at the shorter wavelengths since their stability appears to be greater than that reported by Praetorius.

* Aerated prior to reading.

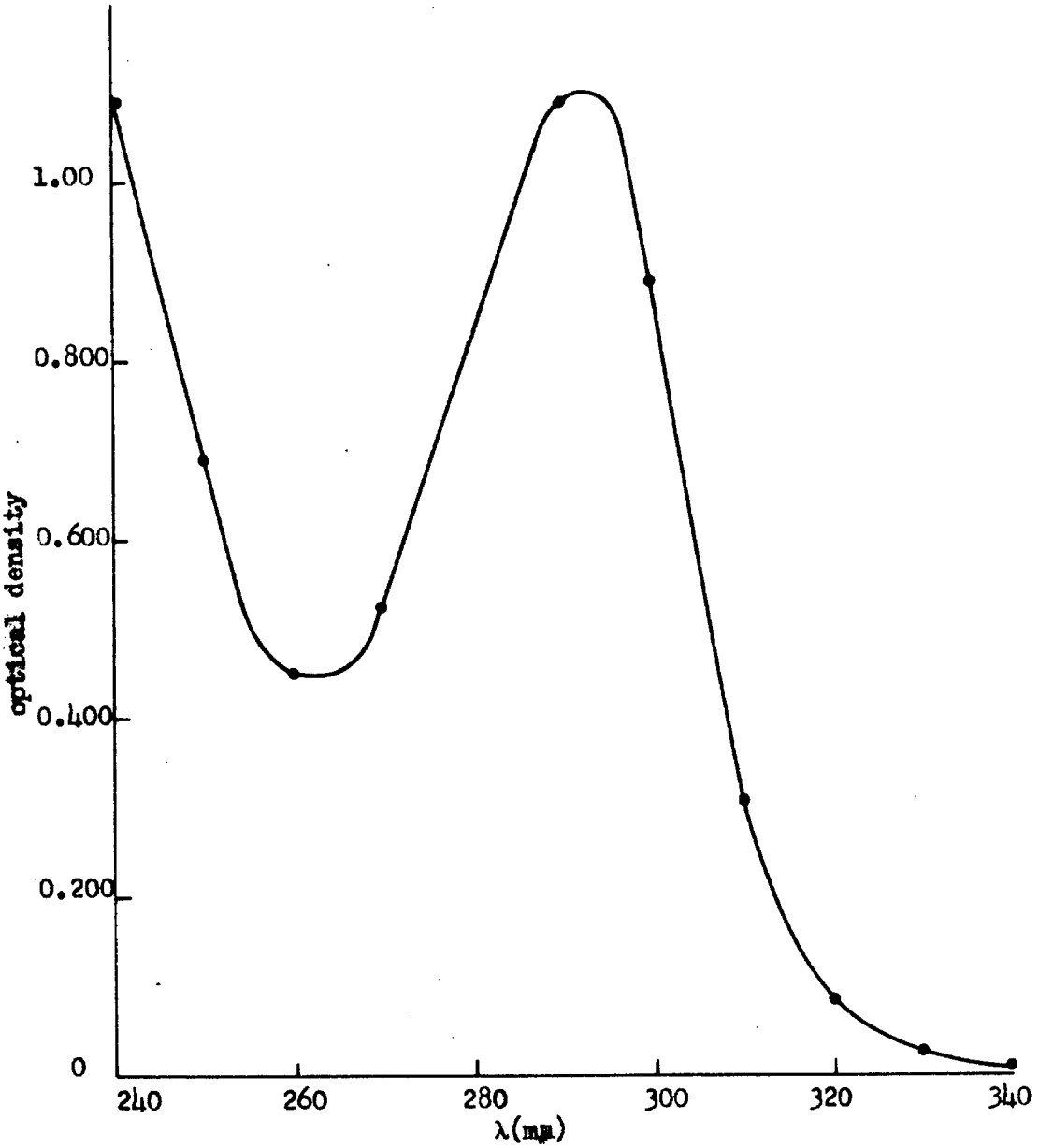


Figure 15 TWO MIN. INCUBATION ABSORPTION SPECTRUM OF REACTION MIXTURE

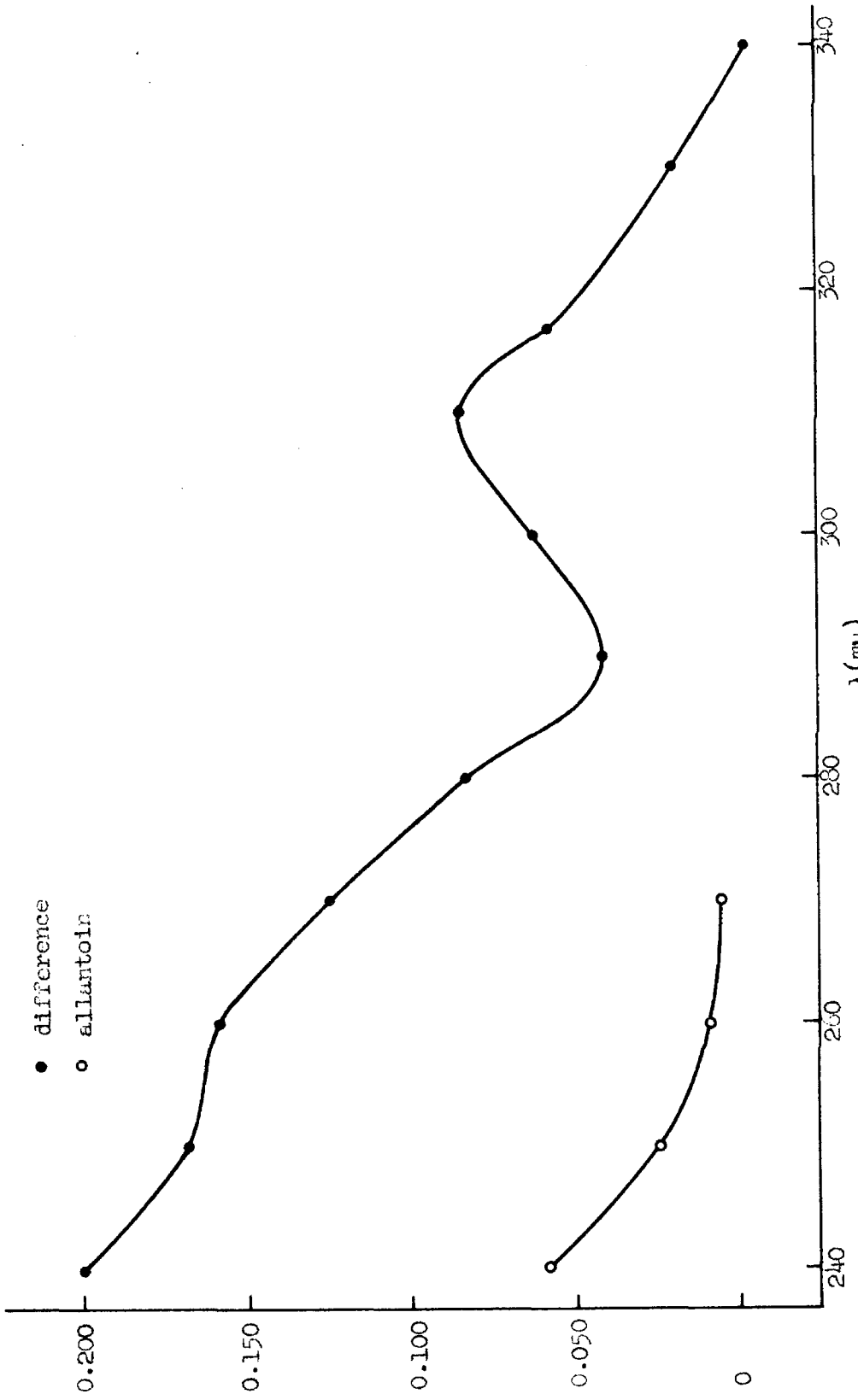


Figure 16 TWO MIN. INCUBATION - DIFFERENCE BETWEEN ABSORPTION SPECTRA OF REACTION MIXTURE AND CYANIDE TREATED REACTION MIXTURE.

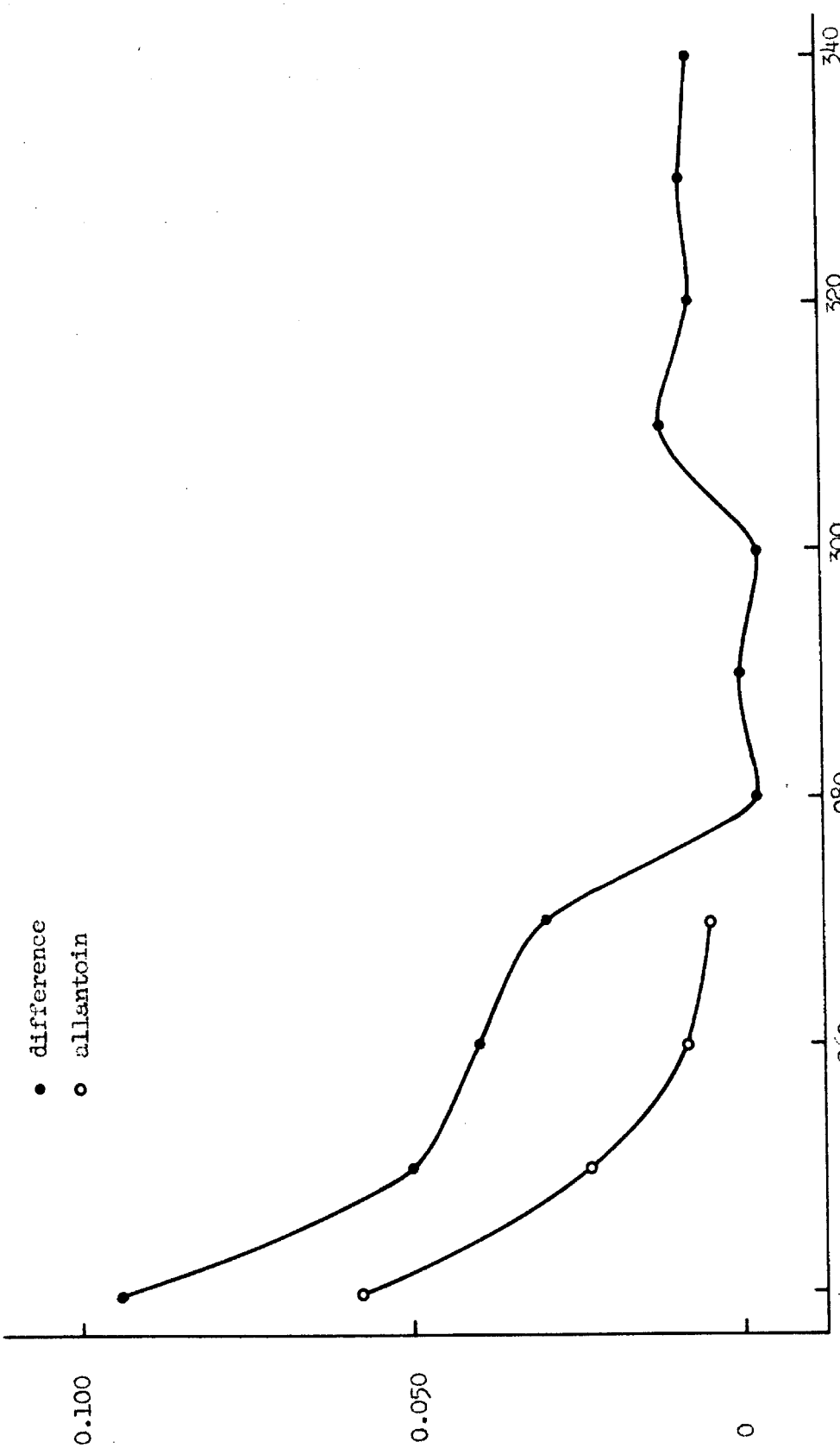


Figure 17 TWO MIN. INCUBATION - DIFFERENCE BETWEEN ABSORPTION SPECTRA OF CYANIDE TREATED REACTION MIXTURE AND URIC ACID.

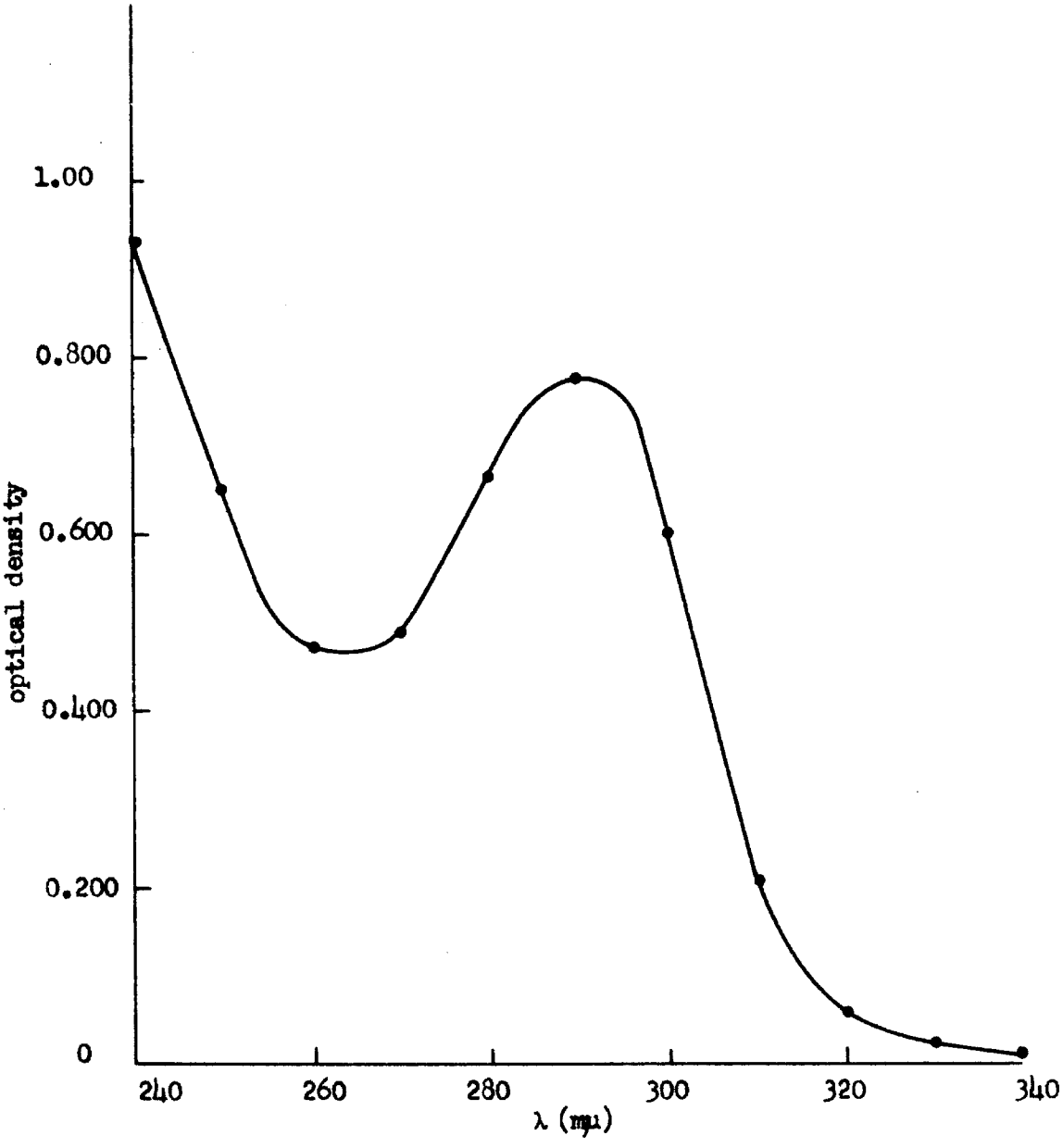


Figure 18 FIVE MIN. INCUBATION ABSORPTION SPECTRUM OF REACTION MIXTURE.

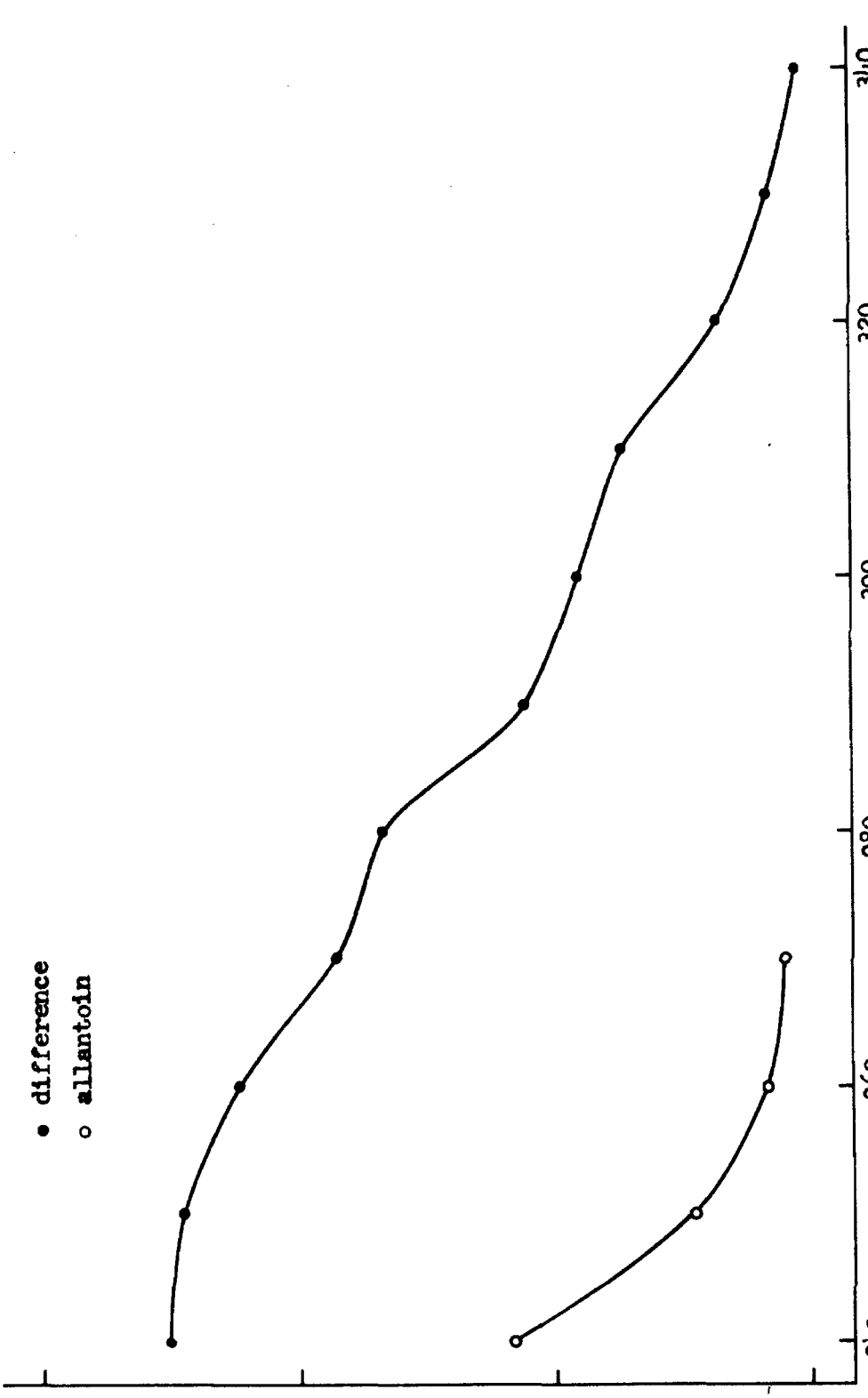


Figure 19 FIVE MIN. INCUBATION-DIFFERENCE BETWEEN ABSORPTION SPECTRA OF REACTION MIXTURE AND CYANIDE TREATED REACTION MIXTURE.

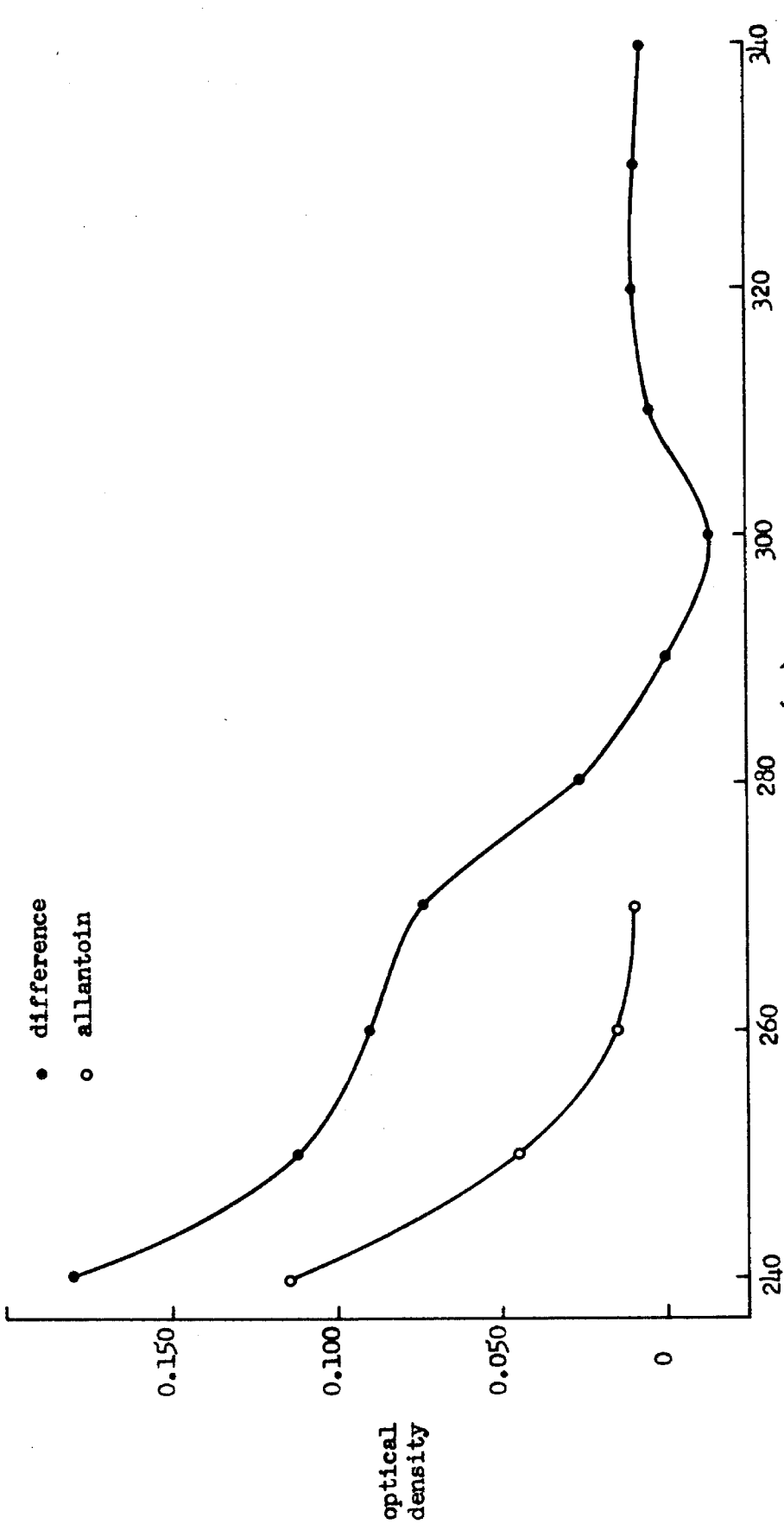


Figure 20 FIVE MIN. INCUBATION-DIFFERENCE BETWEEN ADSORPTION SPECTRA OF CYANIDE TREATED REACTION MIXTURE AND URIC ACID.

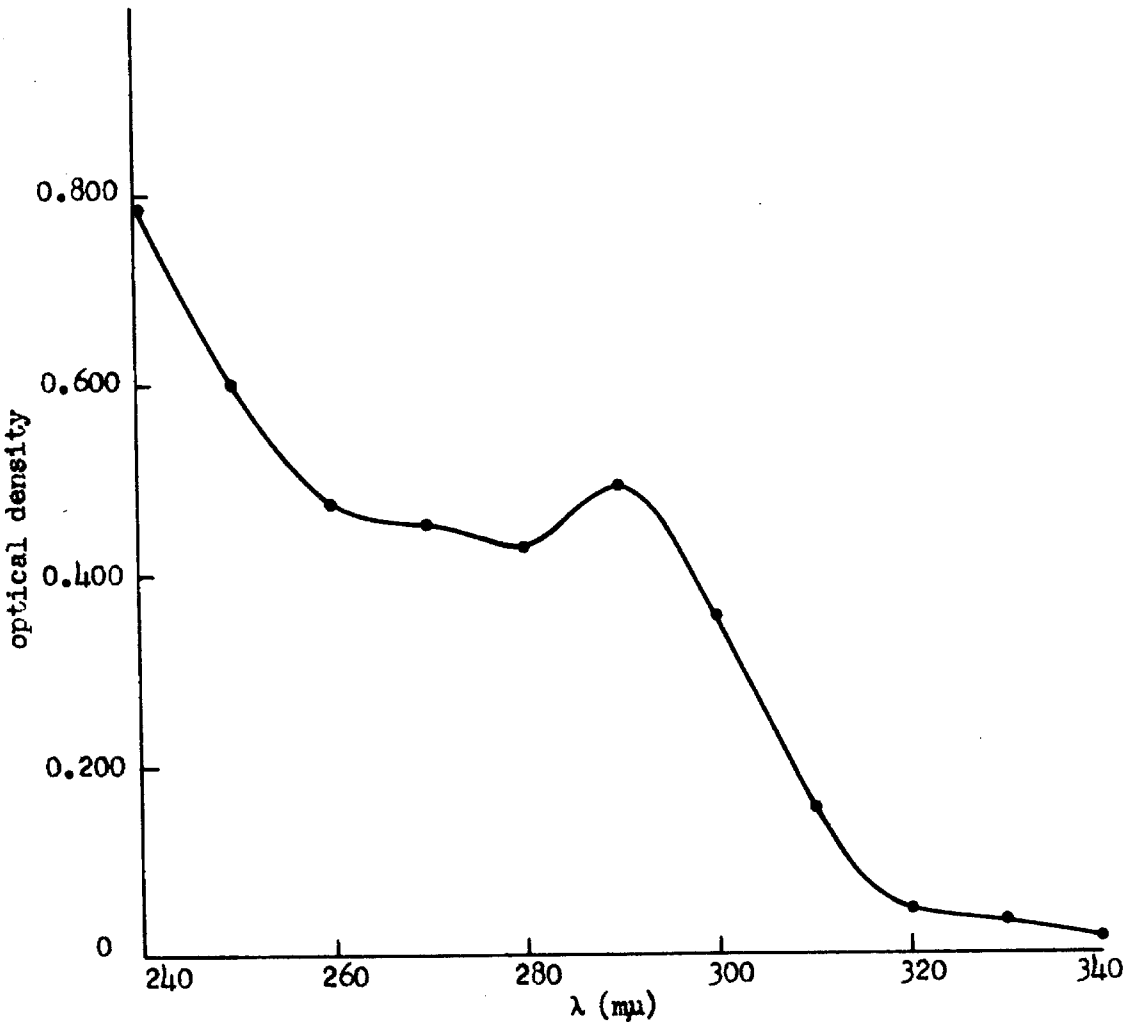


Figure 21 TEN MIN. INCUBATION ABSORPTION SPECTRUM OF REACTION MIXTURE.

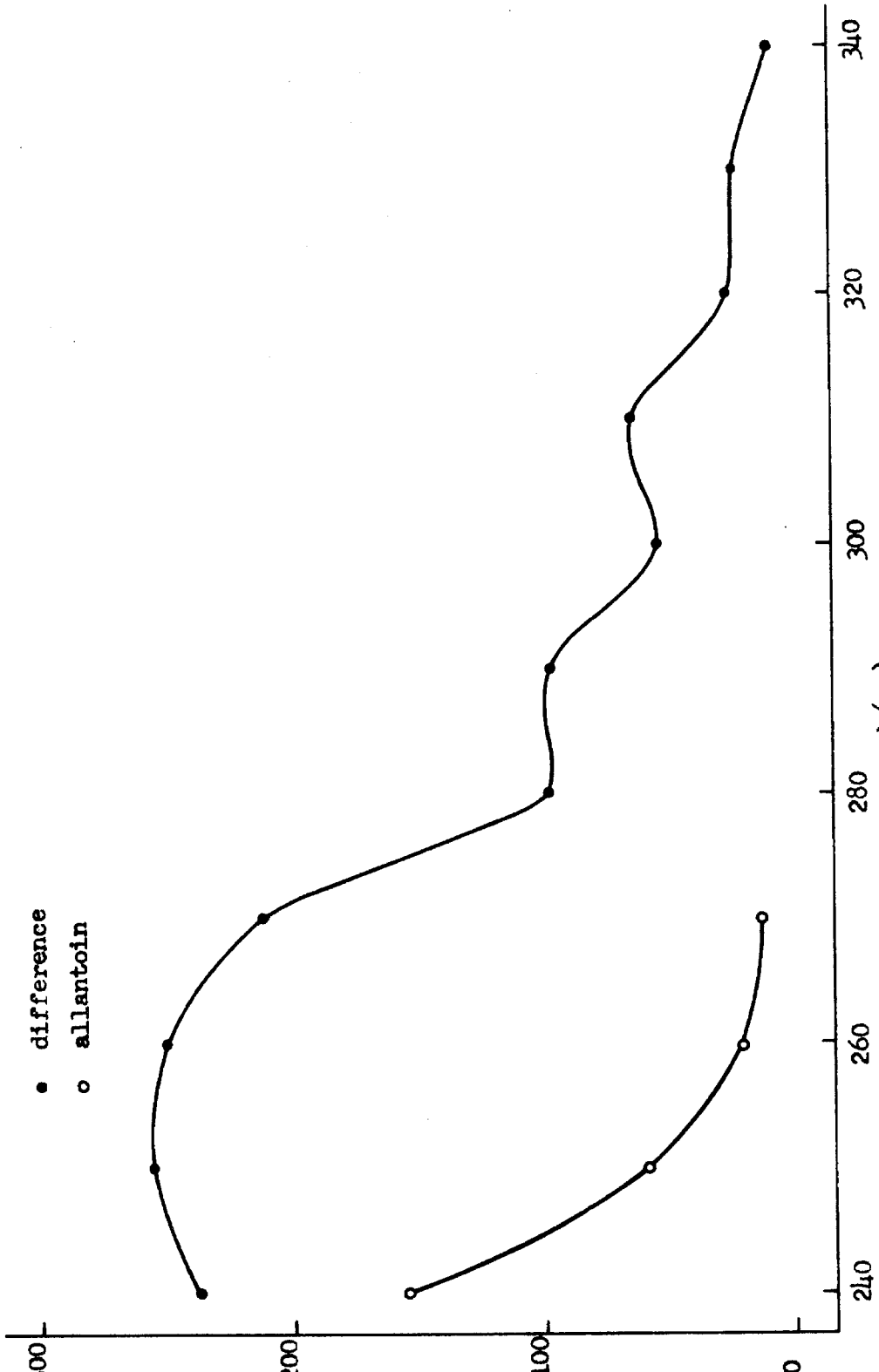


Figure 22 TEN MIN. INCUBATION-DIFFERENCE BETWEEN ABSORPTION SPECTRA OF REACTION MIXTURE AND CYANIDE TREATED REACTION MIXTURE.

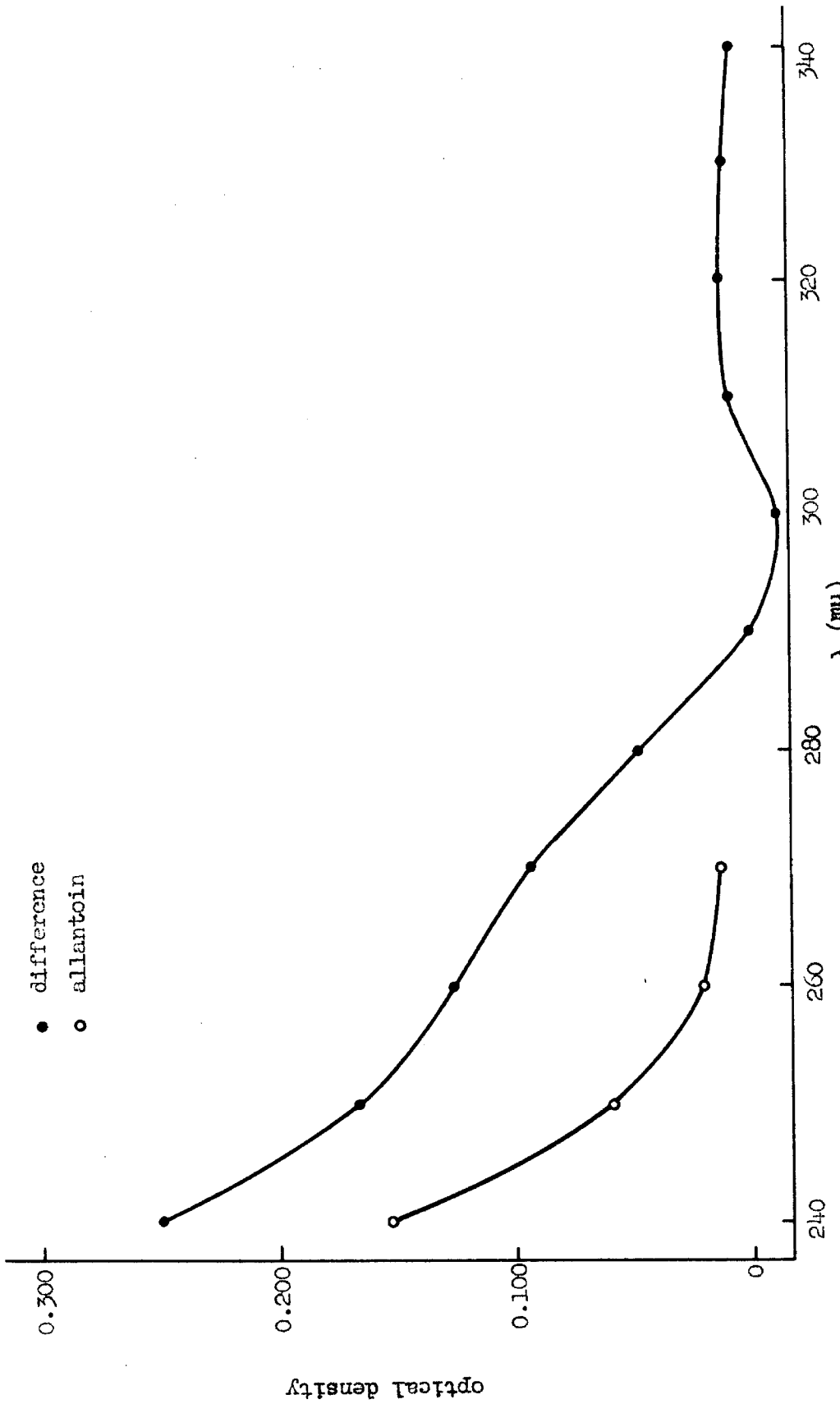


Figure 23 TEN MIN. INCUBATION - DIFFERENCE BETWEEN ABSORPTION SPECTRA OF CYANIDE TREATED REACTION MIXTURE AND URIC ACID

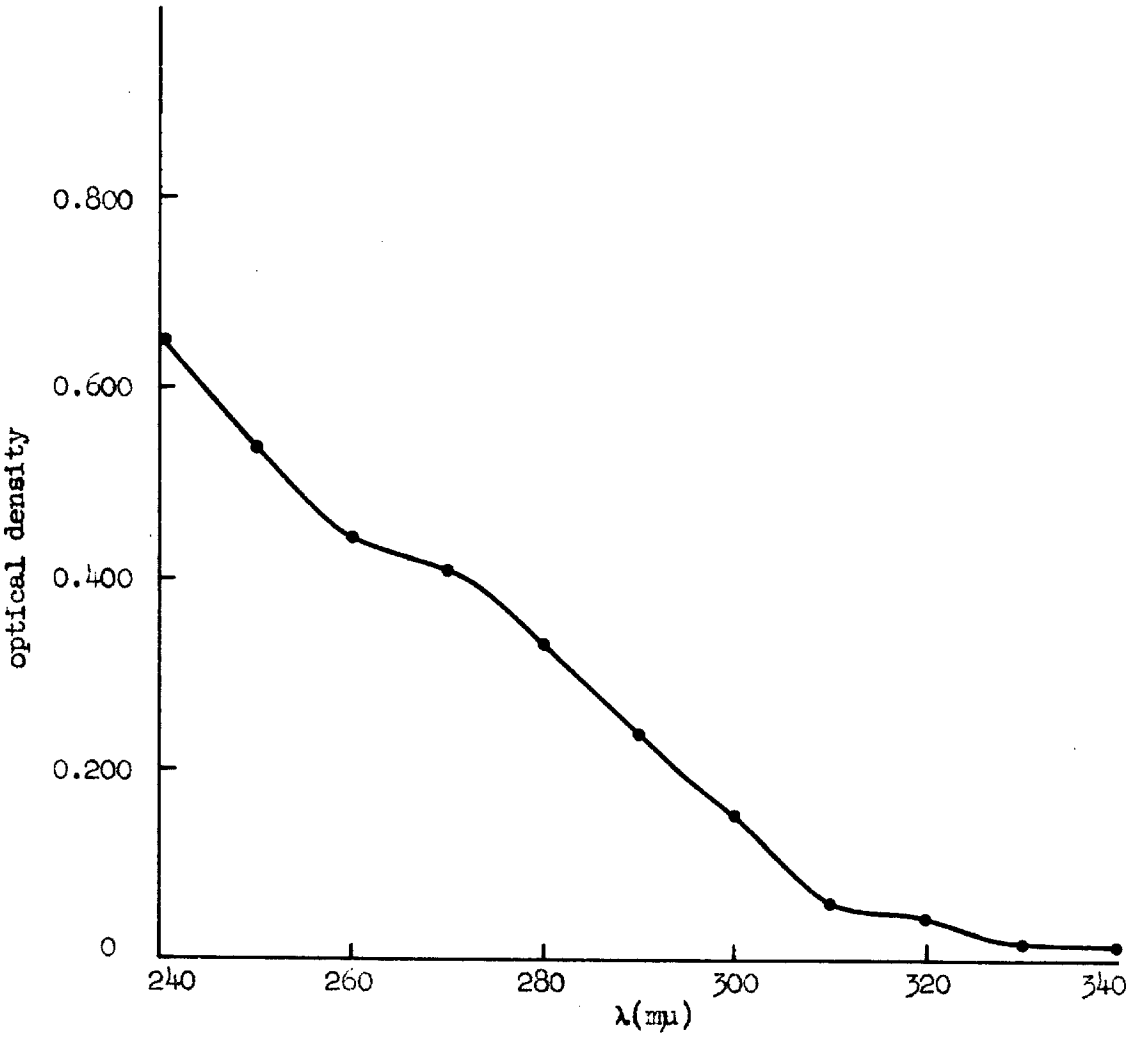


Figure 24 FIFTEEN MIN. INCUBATION
ABSORPTION SPECTRUM OF REACTION MIXTURE

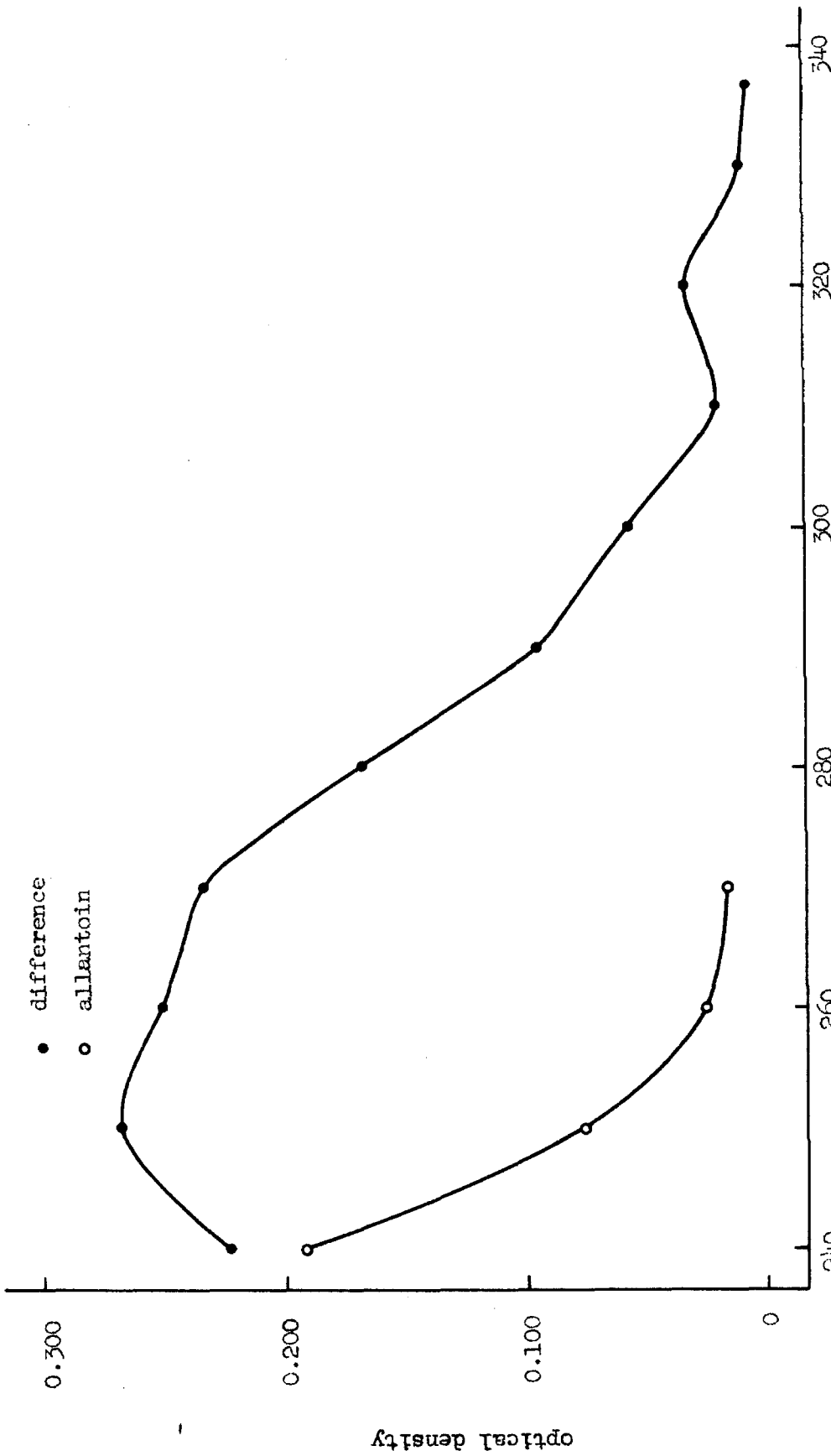


Figure 25 FIFTEEN MIN. INCUBATION - DIFFERENCE BETWEEN ABSORPTION SPECTRA OF REACTION MIXTURE AND CYANIDE TREATED REACTION MIXTURE

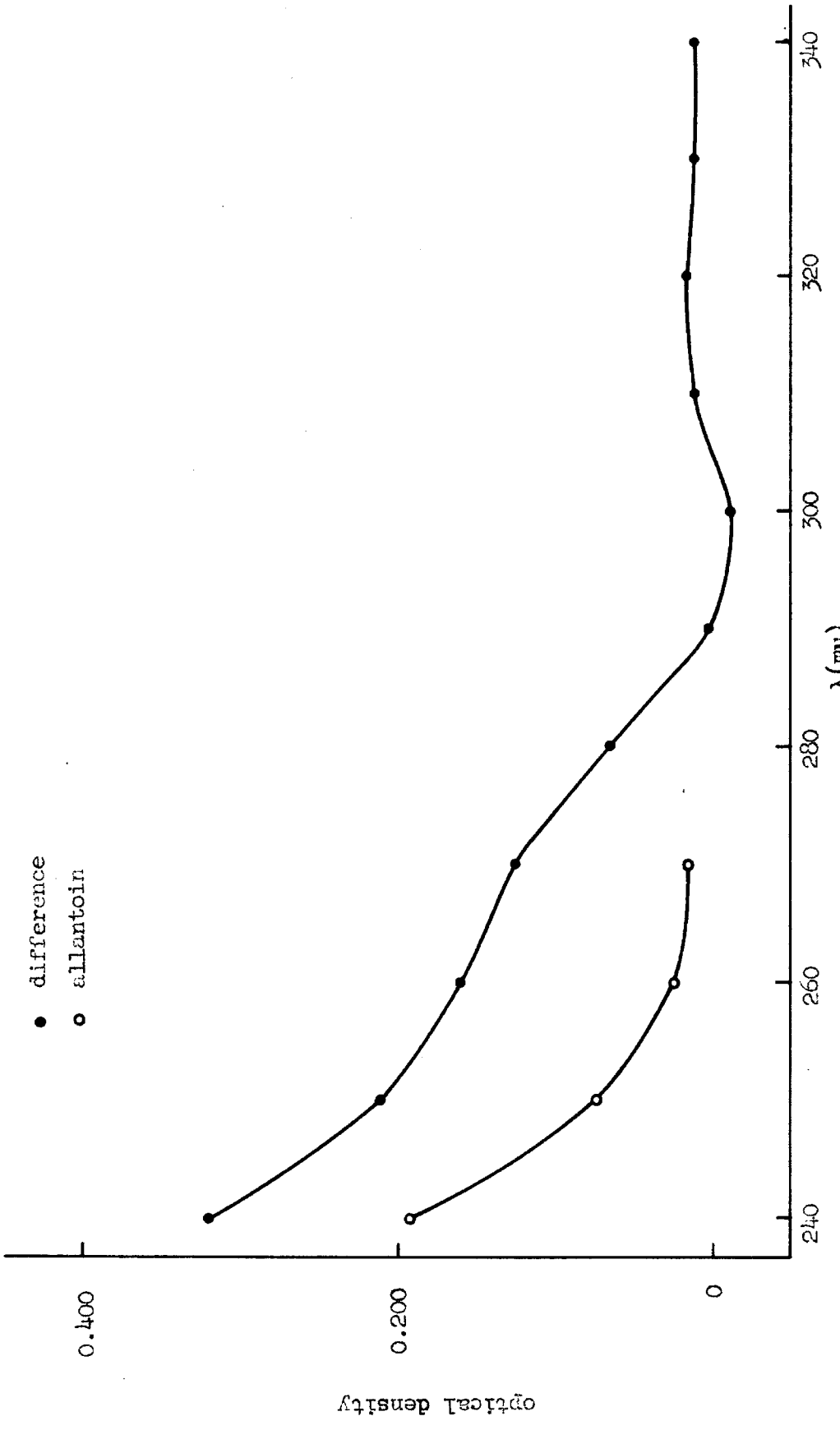


Figure 26 FIFTEEN MIN. INCUBATION - DIFFERENCE BETWEEN ABSORPTION SPECTRA OF CYANIDE TREATED REACTION MIXTURE AND URIC ACID

V. DISCUSSION

For the most part the findings reported in this thesis have been discussed in the sections in which they were presented. Therefore, the only material discussed here is that not covered elsewhere.

The question of whether uricase is a single enzyme or a complex of two or more enzymes still remains unanswered. It is generally assumed, when enzyme activity remains intact through several hundred fold purification, that this activity can be assigned to a single prctein. However this is not necessarily so and some of the stability behavior which was thought to indicate a dissociable cofactor may be due to dissociation of a complex of proteins which cannot be reassociated. In line with this assumption of a single enzyme it has been generally thought that the decay of the immediate products of uric acid oxidation was entirely spontaneous. Since these products are undoubtedly unstable it would be difficult to show their enzymatic destruction. Nevertheless, an indication that catalytic destruction may be occurring is found in the observation of Griffiths (48) that the intermediate absorbing at 325 mμ seemed to be much more stable in his inorganic catalysis system than it is in the enzymatic system of Praetorius (46) or for that matter in the system described in the present study. It is not yet certain that the same compound is involved, but further work with still more highly purified enzymes should provide a solution to this problem.

As has been mentioned before, the problem of biological utilizability of the energy released by the uricase catalyzed reaction is one which remains practically untouched. Whether some phosphate uptake mechanism is operative, such as that suggested by Bentley and Neuberger

(47), could be tested initially by determining P/O ratios of whole tissue preparations with uric acid as the only substrate. If positive results were obtained attempts should be made to isolate a kinase and show that phosphate uptake actually occurs as a result of uricase action rather than at some later oxidation of compounds derived from the glyoxylic acid produced by uric acid degradation. If no phosphorylation occurs it does not necessarily mean that no utilizable energy is obtained, but unfortunately there is no general method for testing other means of energy utilization and further investigations would require another approach.

In general, the state of knowledge of the nature and mechanism of the uricase reaction is far from satisfactory and a large amount of work will have to be done before all the problems are solved. The most promising lines of investigation appear to be continuation of the spectrophotometric studies of the mechanism of the reaction, the isolation and identification of the intermediates indicated, and preparation of a pure uricase for study of its active groups.

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VII. APPENDIX

During the course of these investigations all anionic buffers (phosphate, borate, cacodyllate and pyrophosphate) were prepared as sodium salts except where designated otherwise in the text of this thesis. Sequestrene was used as the trisodium salt. Tris-hydroxy-methyl-amino-methane was used as the hydrochloride.

The earlier workers cited in this thesis did not report the cationic constituents of their buffers.