STUDIES ON THE ACTIVE SPECIES OF MUSHROOM TYROSINASE

by

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TO

My Husband

for his Patience and Assistance

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INTRODUCTION

A. GENERAL INTRODUCTION

The first living cell probably possessed three basic, interdependent, biochemical processes: metabolism, either autotrophic or heterotrophic, energy production, and information transfer (25). The agents which mediated these reactions were the proteins, in particular, the enzymes of this cell. In this hypothetical cell, these enzymes, determined by one gene and mediating one particular metabolic reaction, were controlled by the intrinsic kinetic constants of the reaction they catalyzed. The direction of these reactions--either forward-substrate to product, or reverse-product to original substrate--were dependent upon the relative concentrations of the reactants (25). Gene duplication and independent evolution of genes may have led to separate enzymes, each catalyzing either the forward or the reverse reaction. From this reaction specificity, through mutations and environmental selection, the specificity of individual enzymes may thus have developed (25).

B. ENZYME CONTROL MECHANISMS

As the complexity and specialization of the cell has increased, the necessity for exerting control over its metabolic processes has also increased. The control of skin pigmentation is a potential field for investigation with social consequences which need no explanation in our day. The pigmentation of skin and hair results from melanin, a brown polymer arising from the amino acid, L-tyrosine (59). Although the final steps of melanogenesis are believed to be non-enzymatic in nature,

the catalysis of the first two steps of its formation are mediated by the enzyme, tyrosinase (59). Therefore, an elucidation of the factors affecting the enzymatic activity of this key enzyme and the mechanism of its action presents problems the answers to which could affect the course of history. Medical problems arising from the absence or the unbridled action of tyrosinase include cases of albinism and of melanotic cancer. A more basic understanding of the control mechanisms which affect this enzyme underlies our approach to the problem which will be discussed in this thesis.

There are three possible methods by which cell metabolism may be controlled. These are enzymatic mechanisms, supraenzymatic mechanisms, and genetic mechanisms (74). Included in the category of enzymatic mechanisms are the characteristics of the enzyme and of the reaction it catalyzed such as the turnover number, reaction rates, and co-factor requirements. Obligatory coupling into enzyme systems and the competition between these systems for single metabolites are two other potential enzymatic control mechanisms. The fine control exerted by metals and allosteric reactants on reactions mediated by constitutive enzymes of more than one subunit is another type of enzymatic control (74). Supra-enzymatic mechanisms include the localization of enzymes or enzyme systems within the cell or subcellular particles. Regulation was thus afforded by transport and diffusion of small metabolites. Genetically, metabolism was controlled by structural and regulatory genes responsive to challenges from substrates, hormones, and vitamins (74).

A mutation, substitution or deletion in the genome of the cell may have caused an increase in the proportion of hydrophobic residues in the

enzyme by substitution of non-polar residues for polar residues. This alteration in protein composition allowed the aggregation of subunits into a polymeric protein. The potential for metabolic control of that new enzyme influenced its incorporation into the genotype of the cell. Further mutations might have produced a single enzyme out of a polymeric protein. For example, tryptophan synthetase is composed of two subunits in *E. coli* while in *Neurospora* it is a single peptide chain (25). However, enzymes made up of aggregates of subunits appear to be important loci of control mechanisms which are mediated by allosteric interactions and could have been naturally selected for that reason (25).

C. ALLOSTERISM

The allosterism of proteins allows regulation of their enzymatic activity by small metabolites called ligands. These metabolites, some of which are modifiers, such as inhibitors or activators, bind with a protein at a locus which may or may not be the active site (64). This regulation is accomplished by indirect interactions between the subunits of proteins. A non-allosteric protein exhibits Michaelis-Menten kinetics, that is, the observed velocity of the enzymatic reaction at a given enzyme concentration is, within a limited range, proportional to the substrate concentration (93). A rectangular hyperbolic curve is thus produced on which the enzymatic reaction approaches a maximum at saturating substrate concentrations (93). For allosteric enzymes, the graph of reaction velocity vs substrate concentration has a sigmoidal shape indicative of interactions between the active sites (64, 65).

Two general properties of allosteric proteins are that they are polymers composed of several subunits which may or may not be similar and that allosteric interactions can be correlated with some alteration in the quaternary structure of these proteins (64, 65).

Allosterism was first observed in hemoglobin as heme-heme interactions (11). The affinity of each heme for oxygen is altered after a single heme has been bound to oxygen (22, 88). With the elucidation of the structure of hemoglobin, the hemes were found to be separated from each other in the protein (79). It was necessary, therefore, for the observed interactions to have been mediated by changes in the protein's quaternary structure.

Until the early 1960's, hemoglobin was an almost unique example of allosteric transition (65). However, these transitions have now been observed in a variety of proteins, including enzymes. To date, 50 to 100 proteins have been shown to have subunits (6). Many investigators have sought to explore these polymeric proteins for evidence of allosteric interactions and to offer theories to explain the observed kinetics.

1. Subunit Interactions.

One opinion, that held by Monod *et al.* (64, 65) and by Koshland *et al.* (52), assumes that interactions between subunits in a polymeric protein are sufficient by themselves to account for an allosteric kinetic curve. Other theories suggest that a loose association between subunits permits a dissociation-association equilibrium between more and less active forms, and that this aggregation phenomenon produces the observed

allosteric effects. Such a theory is advanced by Frieden (30) and by Nichol *et al.* (73).

The simplest proposal, that suggested by Monod *et al.* (64, 65), describes an equilibrium between protein conformational states independent of any ligand in the environment. At least one of these states, designated by him as the "R" state, readily combines with substrate, while another state, the "T" state, binds more strongly with the inhibitor. These transformations are symmetrical, that is, all of the subunits of a polymer transform simultaneously from the T state to the R state, in spite of possible substrate molecules at the active site (64, 65).

A kinetic curve may be derived for hemoglobin which agrees with experimental data, as well as conforming with the above restrictions (64, 65). Recently, Gerhart and co-workers have related the kinetics of the enzyme aspartate transcarbamylase to the Monod model (18, 19, 33). Studies using equilibrium dialysis, spectrophotometry and the ultracentrifuge were employed by these workers to determine the number of binding sites for substrate and for inhibitor per subunit and per tetrameric polymer (18, 33). These methods also produced evidence pointing to a conformational change within the polymer upon binding to the substrate and to the modifiers (18, 33). A simultaneous transition between R and T states was assumed because these conformational changes were observed when only 15% of a saturating concentration of substrate was present (33).

Koshland and co-workers feel that the simple model of Monod does not adequately explain all the accumulated data about hemoglobin. An

extension of Monod's theory to the more complicated interactions observed in other proteins is difficult (52). Koshland visualizes a more flexible model based on the principle of induced fit. Introduction of a ligand into a protein produces a deformity observed as a conformational change in that subunit, and alters the stability and the shape of neighboring subunits (52). This is induced fit. According to Koshland's theory, the affinity of the adjacent subunits for the ligand molecule is indirectly modified by the initial deformation. Each additional ligand also affects the conformation and stability of the remaining, non-combined subunits. From the shape of the kinetic curve, the strength of coupling between subunits in a polymer can be observed. Loosely coupled subunits will give Michaelis-Menten kinetic curves, although they still may undergo conformational changes upon binding with substrates (52). Sequential changes which lead to hybrid conformational states are permitted by this model (52).

Results with hemoglobin labelled by coupling with a compound having a known electron paramagnetic resonance, seemed to imply a conformational change which accompanies the binding of each oxygen to the protein (52). Thus, although the observed kinetics are adequately explained by either above mentioned theory of interaction, the bulk of the evidence with hemoglobin seems to suggest an induced fix mechanism of sequential transformations (52). Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, a tetrameric enzyme, exhibits a negative homotropic effect (64, 65) so that the binding of the first ligand restricts the ease with which a second similar ligand can be bound. Such an effect can not be resolved by the symmetry model, but requires

the supposition of sequential changes to explain all the data (52).

2. Association-Dissociation.

Still, for the best known subject, hemoglobin, the proposed models do not account for all the experimental data. There are documented examples illustrating that the oxygenation of hemoglobin weakens the intersubunit bonds, and produces a dissociation into dimeric units (4, 9, 30, 58, 73).

An attempt to expand the theories presented above to include the phenomenon of dissociation-association was made by Frieden (30). He assumed that the changes in conformational states represent a difference in the degree of polymerization rather than simply a steric change in the polymer. To produce allosteric kinetics from this model, the rate of association-dissociation must be rapid compared to the overall rate of the enzymatic reaction. The presence of an allosteric ligand and the concentration of the total enzyme both influence the equilibrium constant of this dissociation. A normal kinetic behavior over a large range of ligand and enzyme concentrations would be possible if a concentration-dependent aggregation was assumed for this system (30).

Glutamic dehydrogenase (GDH) is presented as an example of an enzyme whose known properties are adequately explained by Frieden's model (30). The presence of inducer, adenosine diphosphate (ADP), and coenzyme, reduced nicotinamide adenine dinucleotide phosphate (NADPH), enhance the association of this enzyme to the tetrameric form (29). In dilute concentrations such as those used for enzyme assay, light scattering studies imply the existence of an active monomer. The presence

of inhibitor, guanosine triphosphate (GTP) and coenzyme, NADP, favor the dissociated form of GDH and give rise to combined allosteric activity at enzyme levels where there is some degree of association (29, 30). Small molecules, such as ADP and GTP, affect the association-equilibrium in such a way as to establish the predominance of one of the polymerization states, either monomer or tetramer (29). In the case of GDH, therefore, both the R and the T states are active, but the tetramer is the preferred R state in the presence of substrate, coenzyme and activator (30, 64, 65).

The most comprehensive treatment of allosteric systems is that by Nichol and co-workers (73). Starting from the extremely general example of a polymer interacting with lower association forms, each of which has a different number of binding sites for ligand, and ranging all the way to proteins with equivalent sites on a single macromolecule, Nichol's theory includes and expands upon all of the aforementioned theories. Equivalent binding sites are assumed on all the polymers at each level, with preference for a site shown by the ligand on a weight-concentration basis. The predominant aggregation state in the equilibrium mixture binds ligand more strongly because there is more of it. Allosteric kinetics are also observed when one polymerization species binds the ligand more strongly than another species due to a loss or gain in the number of binding sites after aggregation. The absence of sigmoidal kinetics is noted in cases where the binding constants, the concentrations, and the number of binding sites are all equal for all the species in the polymerization equilibrium.

Nichol's model is expanded to include hemoglobin, following the reasoning of Benesch *et al.* (9) who noted that the observed kinetics of hemoglobin must result both from subunit interactions in the dimer (52), and from association-dissociation between the dimer and the tetramer (73). An interesting enzyme which serves to illustrate this theory is the *delta*-aminolevulinic acid dehydratase from *Rhodopseudomonas spheroides*, which catalyzes the production of porphobilinogen, the precursor of the porphyrins, from *delta*-aminolevulinic acid (68, 69). In concentrated solutions free of monovalent cations, it exhibits the characteristics of an allosteric enzyme, while in dilute solutions in the presence of monovalent cations, it exhibits Michaelis-Menten kinetics. Sedimentation studies in sucrose density gradients containing monovalent cations show an equilibrium mixture of monomer, dimer, and trimer association states. In the same studies, omitting monovalent cations, only the monomer is present. If the substrate, *delta*-aminolevulinic acid excluding monovalent cations, is included in the gradient, the association mixture contains a predominance of monomer. The total activity of the enzyme seems to be related to the relative distribution of each component of the equilibrium mixture, which in turn is a reflection of the state of activation of the monomer. Another consideration in these studies is that the speed of equilibrium between the three states is relatively slow, since they are easily separated by centrifugation in a sucrose gradient (68, 69). Therefore, under the most activating conditions, the speed of the association equilibrium is inadequate to give rise to an allosteric kinetic curve (30).

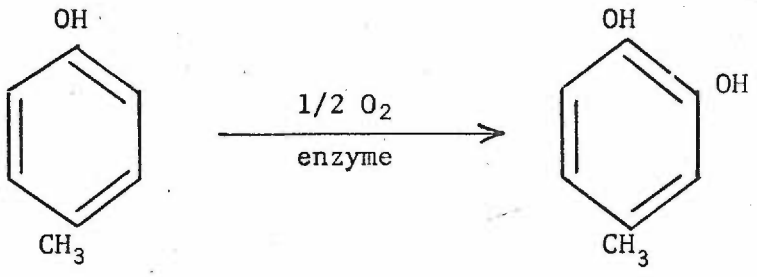
Other examples of an active dissociation species are: rabbit muscle aldolase, a tetramer less active than the dimeric state as determined by Sephadex chromatography (94); methionyl-t-RNA-synthetase from *E. coli*, a tetramer having a more active monomer, as determined by sedimentation analysis (17); haemocyanin, analogous to hemoglobin, of which the monomer is favored in the oxygenated form (66) rather than the dimer as in hemoglobin (9, 57); amino acid oxidase, which exists in an equilibrium mixture of monomer, dimer, and trimer, and the actual activity of any species has not been determined (1, 40); and phosphorylase b, a tetramer which, although having an active dimer, is more active in its associated form (10).

D. TYROSINASE

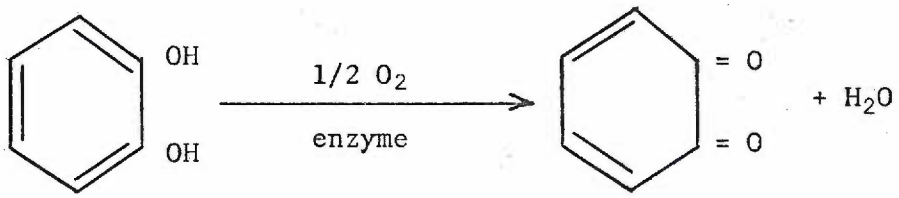
1. Function.

Tyrosinase catalyzes the conversion of tyrosine to o-dihydroxyphenylalanine-quinone (DOPA-quinone). DOPA-quinone then condenses, through a series of non-enzymatic reactions, to melanin (59), a pigment of hair and skin. Although plant tyrosinase oxidizes many mono- and diphenols, the mammalian enzyme is quite specific in oxidizing only L-tyrosine and L-DOPA (59).

Tyrosinase is unique in that it catalyzes two different reactions. The first, referred to as the "cresolase" activity of tyrosinase, is the conversion of a monophenol to its corresponding o-quinone. The term "cresolase" derives from a reference to p-cresol which is commonly used in the laboratory to test for hydroxylating activity (71):



The second reaction catalyzed by tyrosinase is the dehydrogenation of o-diphenol to the o-quinone. As catechol is a readily available substrate for this reaction *in vitro*, this function of tyrosinase is known as its "catecholase" activity (71):



It should be noted that catechol as a substrate is far from ideal, as a pronounced inactivation or destruction of the enzyme occurs during the initial stages of its oxidation (24, 70, 71). Early attempts to credit this destruction to the o-quinone have failed (71). This inactivation, therefore, was believed to be a direct consequence of the catalytic combination of enzyme, catechol, and oxygen (71) resulting in a nucleophilic addition of the protein to the o-quinone of catechol and a release of the copper from the enzyme (14). Other catecholase substrates, such as 4-*tert*butylcatechol and 3,4-dimethylcatechol, do not produce reaction inactivation (71). All attempts to relegate the cresolase and catecholase activities to two different enzymes have failed (53, 55).

Tyrosinase was isolated from mushroom extract by Bourquelot and Bertrand in 1883 (47). Indeed the term "oxydase" was coined by Bourquelot to describe this pigment forming enzyme (61). Bertrand isolated and crystallized from the mushroom extract the natural substrate of his "oxydase". This he identified as L-tyrosine, and introduced the term "tyrosinase" in referring to this enzyme (47).

2. Mechanism.

Much of the early work on tyrosinase centered on its mechanism of action. The two reactions mediated by the enzyme present a rare phenomenon, possible explanations of which have been the subject of much debate. Here are a few examples of the theories which have at some time been in vogue: in 1908, Bertrand proposed that tyrosinase consisted of a specific peroxidase as well as an oxygenase (47):

$$\text{Tyrosine} + \text{"active } O_2\text{"} \longrightarrow \text{DOPA} \xrightarrow{\text{oxygenase}} \text{peroxide} + \text{products}$$

$$\text{Peroxide} + \text{peroxidase} \longrightarrow \text{product} + \text{"active } O_2\text{"}$$

The oxygenase could be replaced by hydrogen peroxide and the enzymatic activity retained (47):

$$\text{DOPA} \xrightarrow{\text{autooxidation}} \text{peroxide} + \text{products.}$$

Onslow in 1923 postulated that the tyrosinase complex consisted of a water splitting enzyme (a reductase or deaminase) and an oxidase (75):

1. Tyrosine + H_2O $\xrightarrow{\text{deaminase}}$ p-hydroxylphenylacetaldehyde + CO_2 + NH_3 + H^+

2. a. tyrosine + traces of "active O_2" $\longrightarrow$ DOPA

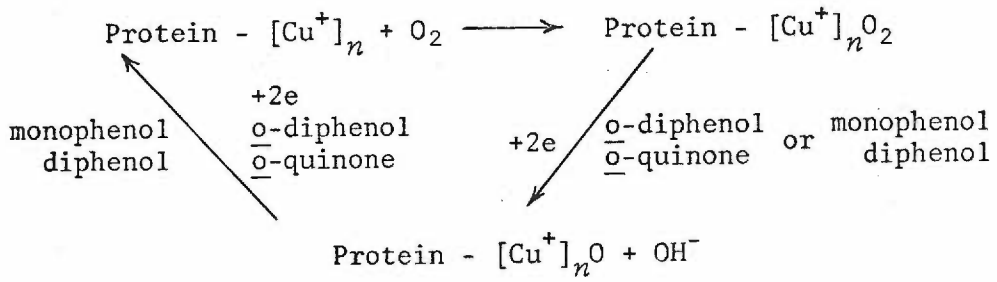
   b. DOPA + oxygenase $\longrightarrow$ peroxide + oxidation products

   c. peroxide + peroxidase $\longrightarrow$ "active O_2"

The hydrogen produced by sequence 1 acts as an acceptor for the active O_2 produced in the second sequence (75). Her later hypothesis concerning the nature of the "phenolase complex" is accepted by some investigators today (95). She proposed that tyrosinase is only a polyphenol oxidase. Any monophenolase activity formerly attributed to the enzyme is, in her opinion, actually the result of catalysis by the o-quinoid products of the enzymatic reaction (76).

Nelson (71, 77) and Mason (60) have attributed both cresolase and catecholase activity to one enzyme. That the mechanism proposed by Onslow is incorrect is implied both by the inability of other o-quinones to hydroxylate monophenols, and by the specificity in the hydroxylation reaction for the ortho position of the monophenol (60, 62). The strongest evidence in favor of the polyphenolase scheme of the Onslow hypothesis is that, when monophenols have been used as substrates, a lag period occurs before the hydroxylation reaction begins (34, 62). However, this lag period is quickly dispelled by the addition of diphenol or other reducing agents to the reaction mixture (62), and O^{18} studies with H_2O^{18} and $O_2{}^{18}$ have shown that the oxygen introduced into the phenol

comes from the molecular oxygen of the air, rather than from water as would be necessary if the Onslow scheme were correct (63). These facts have led some investigators to propose simultaneous hydroxylation of a monophenol and dehydrogenation of a diphenol (36, 59). Recent studies suggesting that tyrosinase is a mixed function oxidase requiring catechol for its operation have reinforced this supposition (62, 95). Incorporation of all these facts into a mechanism for tyrosinase action produces the scheme proposed by Mason (61):



3. Physical Complexities.

In recent years the problem has been further complicated by reports from various investigators of the presence of tyrosinases with similar as well as dissimilar subunits in purified preparations (28, 43, 78, 82). In the case of mushroom tyrosinase, this multiplicity of structure can be attributed, at least in part, to a monomer-tetramer equilibrium (42). In the association equilibrium, the tetrameric form seems to predominate (43).

When a purified preparation of mushroom tyrosinase is chromatographed on hydroxylapatite, several fractions, called isoenzymes or isozymes, are eluted. These isozymes differ in their catecholase-cresolase ratios, but three have similar amino acid compositions (12,

45, 86). Each of the isozymes, as examined by acrylamide gel electrophoresis, shows evidence of a monomer, dimer, tetramer association equilibrium (42, 43, 44).

4. Active Site Studies.

The active site of tyrosinase contains copper (53, 55). In work with the enzyme extracted from potato, copper was removed and all enzymatic activity was lost. With the addition of copper and reduction to the cuprous form, the activity was regained (55).

The copper of tyrosinase is known to bind carbon monoxide in the presence of substrate (37, 53). Kubowitz found CO to Cu ratio in the presence of catechol to be 0.5 (55). Therefore, all reaction mechanisms postulated for this enzyme up to the present time have assumed there to be two copper ions per each active site (13). In a recent attempt to confirm this work, radioactive carbon monoxide was bound to the copper of the enzyme in the presence of both a monophenol and a diphenol substrate. The CO/Cu ratio was 0.5 when p-cresol was the substrate, and 1.0 when catechol was the substrate (37). This suggests that, in the presence of monophenol, tyrosinase is active in its aggregated form, i.e. tetramer or dimer, and, in the presence of catechol, it is active as a monomer (37).

E. STATEMENT OF THE PROBLEM

With the preceding as our foundation and our inspiration, we have sought to determine what effect its substrates may have upon the state of aggregation of tyrosinase. We investigated the effects of different

substrates, of substrate concentration, and of oxygen content in the experimental environment. Two approaches were used in this work. We employed the band centrifugation technique to determine the sedimentation coefficient of the active complex in the presence of substrate. After these experiments were concluded, a thorough study of the reaction catalyzed by tyrosinase through the medium of Sephadex chromatography was also conducted.

MATERIALS AND METHODS

A. ENZYME PREPARATIONS

One of the reasons for the mechanism controversy lies in the inconsistency of the catecholase to cresolase ratios in the purified products (23). It has been shown that either high cresolase or high catecholase fractions can be obtained from the same enzyme source (12, 48, 86). The ratio of the activities is completely dependent upon the purification scheme. In general, the high cresolase preparations are less stable than the high catecholase preparations, and require more delicate techniques to obtain.

The method used in this work will be published in detail in Methods in Enzymology (72). It is a recombination of steps described by other investigators into a reliable and repeatable procedure for our purposes (12, 15, 48). This procedure is outlined as follows:

Freshly picked commercial mushrooms, *Agaricus bispora*, were frozen overnight at -20° C before the following steps were undertaken. Six weeks were required to complete these procedures.

1. Three pounds of fresh, frozen mushrooms and 3.5 liters of acetone at -20° C were homogenized in a four liter Waring blender at low speed for 1 minute. This suspension was then poured onto a 40 cm Buchner vacuum filter which was covered with a large nylon cap. Whatmann #202 filter paper was used. An additional three pounds of mushroom were then homogenized in a similar manner and then added to the first batch. All six pounds were filtered free of liquid by suction, covered with the nylon cap, and frozen with dry ice. After solidification was complete,

the cake was disintegrated and reblended for 2 minutes in 3 liters of cooled acetone.

2. The homogenate obtained after the second dehydration was filtered as before. Half of the frozen product was blended for 3 minutes in 3 liters of 30% acetone in water (volume for volume) as described above. This mixture was then centrifuged at 6,000 r.p.m. for 15 minutes in a Servall model RC-2 centrifuge cooled to -20°. Together, the six metal tubes for the Servall hold 2,000 ml. The remaining 1,000 ml of extract were kept at -20° for the next run. Centrifugation of the 30% acetone extract from 18 pounds of mushrooms required 3-3.5 hours.

The supernatant recovered after the above centrifugations was diluted 1:2.5 with cooled acetone added slowly with stirring. The acetone was added over 10-15 minutes and care was taken to maintain the temperature of the suspension below 4°. Thirty minutes of settling time at -20° was allowed before the clear supernatant fluid was decanted from the precipitate. The flocculent precipitate collected from all the mushrooms ground in one day was then centrifuged in the Servall for 15 minutes at 6,000 r.p.m., after which it was dissolved in 0.026 M sodium borate buffer at pH 8.5. We found that grinding the precipitate with 5-10 ml of buffer per centrifuge tube in a mortar previously cooled to -20° to be the easiest method of dissolving the enzyme. After a viscous solution was obtained, more buffer was added until the solution poured easily. 450-500 ml of buffer were used to dissolve the extract from 18 pounds of mushrooms. The enzyme solutions were frozen and stored in the freezer at -20°, until all of a hundred pounds of mushrooms was extracted to this point.

4. The enzyme extracts from 100 pounds of mushrooms were thawed and combined. A small quantity of the dissolved protein precipitates as a result of this procedure so that it was found necessary to centrifuge all fractions before continuing. The sediment from this centrifugation was reground with 185 ml of buffer, centrifuged, and the supernatant added to the original solution which was handled as follows:

5. Cold, saturated ammonium sulfate, neutralized with ammonium hydroxide, was added to the solution until 40% saturation was reached. This enzyme solution was stirred overnight and then centrifuged. The precipitate was dissolved in 10 mM phosphate buffer at pH 6.7. The supernatant was then brought up to 52% saturation with ammonium sulfate and treated with stirring and centrifugation as above. The precipitate from this fractionation was dissolved to a volume of 150 ml and the supernatant taken up to 66% saturation with ammonium sulfate. A precipitate was once more collected.

6. The 40-52% primary fraction was then applied to a Sephadex G-25 column and chromatography was done at room temperature. The Sephadex G-25 was allowed to swell overnight in 0.001 M phosphate. A column with dimensions of 8 x 36.5 cm was used to desalt the enzyme solution. The passage of the enzyme through the column was indicated by its dark color. The darker fractions were collected, assayed for activity and pooled. After the enzyme solution had eluted from the column, two other bands emerged, but these were without activity. The column was regenerated by repeated washings with 0.001 M phosphate until the elutent was clear.

7. At this point, the enzyme solution was brown, probably due to the accumulation of pigment from endogenous substrate released during

the purification procedure. Some of this pigment could be removed by precipitation with saturated lead subacetate. Because excess lead subacetate will remove enzyme along with the pigment this operation is very delicate. A maximum recovery of 70% of the enzymatic activity was obtained with this step. Saturated lead subacetate was added dropwise with continued stirring of the sample, allowing 10-15 minutes between additions. The enzyme solution was cooled in an ice bath during this operation. Our main fraction, after G-25 treatment, was 225 ml in volume. To this solution, 8 ml of saturated lead subacetate were added over a period of 2.5 to 3 hours. The lead subacetate solution was prepared by adding a calculated excess of solid lead subacetate to 100 ml of distilled water, stirring it overnight, allowing the lead subacetate to settle (23). This mixture was then centrifuged in the Servall refrigerated centrifuge at 16,000 r.p.m. for 20 minutes. When the pigment removal was completed, the supernatant was pale brown in color. If it were yellow, too much lead subacetate had been added, and the specific activity of the final preparation was greatly reduced.

8. More pigment precipitated if the solution was left at 0° overnight. After centrifugation, the enzyme solution was applied to the G-25 column and the effluent, containing 280 ml of enzyme, from the second G-25 column, was concentrated by ultracentrifugation. This method consisted of centrifuging the sample in the Spinco model L centrifuge for 12-16 hours at 40,000 r.p.m. The rotor was allowed to stop without braking and more than 11 ml of supernatant from the tube containing 12.5 ml of solution was drawn off by needle and syringe. The desired protein was concentrated in the remaining 0.5-1.0 ml.

9. Finally, chromatography on hydroxylapatite of the enzyme preparation was carried out.

This sequence of steps has been standard in our laboratory for several years. It was described in a more complex form by Bouchilloux *et al.* in 1963 (12). Our methodology differs in the following ways: we omitted the continuous flow electrophoresis step, and we preferred the use of Sephadex G-25 to dialysis for our desalting steps. A loss of cresolase activity has been observed both in ours and in other laboratories after dialysis of tyrosinase preparations (77).

As described by Smith and Krueger (85) and Bouchilloux *et al.* (12), hydroxylapatite chromatography separates the enzyme into its four isozymes, designated in this study α, β, γ, and δ (12) (see Figure 1). These differ from each other in their catecholase to cresolase ratios. The *alpha* enzyme, the first fraction eluted from the column, has high cresolase activity. The *delta* fraction has high catecholase activity, and the *beta* and *gamma* are intermediate in their ratios.

The *delta* fraction, as obtained from hydroxylapatite, contains a percentage of inactive protein, electrophoretically distinct from the enzyme. This lower molecular weight component was removable by chromatography on Sephadex G-100. All other fractions, as obtained from hydroxylapatite, sediment as one peak in the ultracentrifuge. The *delta* fraction, as eluted from G-100, is homogeneous by ultracentrifugation, although it appears to be unstable unless stored in concentrations greater than 20 mg/ml. If stored in concentrations less than 20 mg/ml, this fraction loses activity over a period of three months. This observation agrees with the results obtained in other laboratories upon

Figure 1:

The typical elution pattern obtained from an hydroxylapatite column and the identification of the various fractions α, β, γ, δ and ε. The dimensions of the column were 2.5 x 35.5 cm, and 1.5 mg of protein was applied to the column. The dotted line traces the optical density (OD) at 280 mμ against a water blank. The solid line shows the activity measured against 4-*tert*-butylcatechol with the oxygen electrode, graphed as μmoles O_2/min/ml enzyme solution x 10^2.

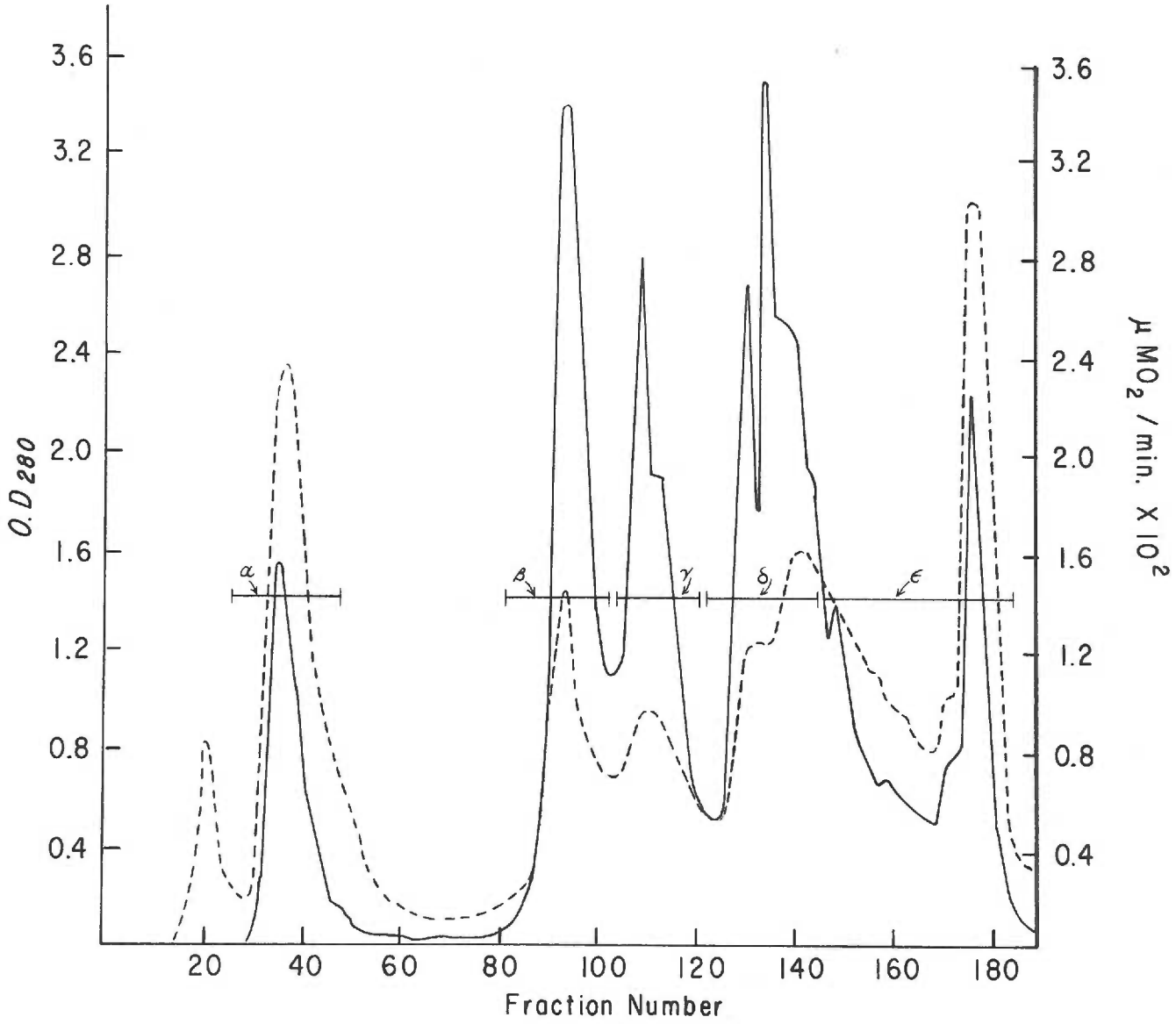

O.D 280
μ MO2 / min. X 10²
Fraction Number
α
β
γ
δ
ε
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
20
40
60
80
100
120
140
160
180

Table I:

This table gives the characteristics of the enzyme used in this work. Each of the purification steps and the measurable parameters at each step are recorded. The final product is carefully characterized; its copper content, activity, both cresolase and catecholase, number of mg of each isozyme, and their specific activities are given.

TABLE I

Fraction	Total Protein	Total Catecholase	% Cu	Cresolase/mg	Specific Catecholase
100 pounds mushrooms	109.54 g	5.1×10^6	-	0.583	46.6
Ammonium sulfate cuts					
0-40%	3.8 g	2.6×10^4	-	-	6.8
40-52%	11.6 g	3.2×10^6	-	1.51	275.
52-66%	4.2 g	1.8×10^5	-	-	42.5
Lead Subacetate[1]	7.07 g	1.5×10^6	0.05	0.421	263.
Hydroxylapatite[2]					
α^1	41.16 mg	20,600	0.16	18.89	500.
α^2	69.09 mg	79,625	0.21	22.54	1152.
β^1	22.25 mg	36,905	0.21	13.46	1658.
β^2	43.16 mg	62,000	0.17	20.46	1439.
γ^1	29.10 mg	44,100	0.26	17.07	1515.
γ^2	80.10 mg	86,316	0.11	5.22	1077.
δ^1	14.41 mg	12,779	0.20	4.53	887.
δ^2	125.85 mg	161,000	0.06	4.32	1279.

1. One-half of this fraction was used to obtain the following results with chromatography.

2. These figures represent two separate column experiments.

the removal of the light component (95).

The *epsilon* fraction, shown in Figure 1, was shown to be electrophoretically distinct from the tyrosinase isozymes. It consisted of a mixture of proteins and smaller peptides which as a group possess low but detectable catecholase activity. No further studies have been done with this fraction to date.

By means of acrylamide gel electrophoresis, the predominance of a Band III, the tetramer, is noted in all the fractions. Bands attributable to the other aggregation states are present only as minor components. Although the different aggregation states exist, each isozyme is pure when examined by electrophoresis (45). The characteristics of this preparation are the subject of Table I.

In reviewing Table I, one should note that one-half of the lead subacetate treated fraction was used to obtain the values reported for the two separate hydroxylapatite column chromatographies. The other half of the subacetate fraction was treated in a similar manner, but the results are not included here because that purified enzyme was not used in our work. The *delta* preparation has not yet been chromatographed on Sephadex G-100. In both *delta* results, the light component (see Figure 11) was found to comprise two-thirds of the reported *delta* protein. After removal of this component, the copper content of $delta_2$ was 0.15, with a corresponding increase in the specific catecholase activity.

B. ULTRACENTRIFUGATION

1. General Introduction.

Separation of component particles according to weight, size, and

shape occurs in a suspension allowed to stand. The same force per unit mass is applied to each particle by gravity, but the differences in frictional resistance of the particles result in their separation (35). The same principle applies to the sedimentation of substances in a centrifuge, but the force of gravity is multiplied. This multiplication is proportional to the square of the angular velocity of the rotor and the distance of the sedimenting particles from the axis of rotation (35).

The first analytical centrifuges made use of an absorption optical system with a monochromatic light source (35). The pattern produced on a viewing screen was that of a sedimenting band. Later developments in optics resulted in a system based on changes in the refractive index of the sedimenting solution. This system, referred to as the schlieren optical system, produced a graph of peaks. The concentration of the sedimenting particles and the angle of the schlieren analyzer with reference to the light path control the size and sharpness of these peaks. The plates obtained by the schlieren system are easier to interpret than those of an absorption system (35).

Two measurable parameters, the diffusion coefficient, D, and the sedimentation coefficient, s, are determined in ultracentrifuge studies.

a. Diffusion coefficient:

To calculate the diffusion coefficient, the synthetic boundary cell is used in the ultracentrifuge, or it may be obtained directly by means of the Tiselius electrophoresis cell (84).

b. Molecular weight:

The molecular weight of a macromolecule can be obtained from the diffusion coefficient and the sedimentation coefficient once they

are determined (7, 84); or directly by either the sedimentation equilibrium method or what is called the "approach to equilibrium" method. As described by these methods, sedimentation equilibrium is the concentration distribution of a solute at the equilibrium position. This position is obtained when the movement of solute in the centrifugal direction is counterbalanced by the diffusion of solute in the centripetal direction (84). The equation for determination of molecular weight is (7, 84):

$$M = \frac{sRT}{(1 - \bar{V}\rho)\ D} \qquad (1)$$

where: $\bar{V}$ = the partial specific volume of the solute,

ρ = density of the solution.

c. Sedimentation coefficient:

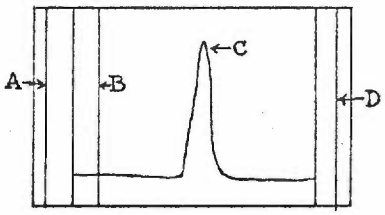
The sedimentation coefficient of a macromolecule is measured by the sedimentation velocity method. This coefficient describes the sedimentation rate of a macromolecule as determined by standard conditions of rotor speed, temperature and solute concentration. The molecular weight of the sedimenting species cannot be calculated from the sedimentation coefficient alone. Other factors besides weight (e.g. shape, and compactness of the particles) influence the sedimenting speed. The popularity of sedimentation velocity studies is attributable to the ease and speed with which the sedimentation coefficient can be calculated. From the sedimentation pattern recorded on the photographic plates, the homogeneity of the macromolecular species in terms of molecular weight can be estimated. The equation for the determination of the sedimentation coefficient is (7, 84):

$$s = \frac{1}{\omega^2 x} \frac{dx}{dt} \qquad (2)$$

where: x = distance from the axis of rotation to the sedimenting boundary,

ω = angular velocity[1].

The chart for calculating the observed sedimentation coefficient is shown in Figure 2. A, B, C and D are values read from the centrifugation plates and correspond to the following points (8):



where: A and D are the reference points and are a specific distance from the rotor's axis of rotation,

B is the meniscus of the solution in the centerpiece,

C is the point of maximum concentration of the sedimenting component.

A microcomparator has been especially designed for this purpose (31). F in columns 13 and 14 is the magnification factor which describes the discrepancy between the distance as recorded by microcomparator, and the actual distance between the reference points. A cell rotating under the influence of a centrifugal force field is caused to change its shape and to stretch. In columns 15 and 16, f_1 and f_2 are correction factors describing the amount of stretching produced by the angular rotation (8):

---

1. This designation holds throughout this thesis.

Figure 2:

This is the form for measuring and calculating the sedimentation coefficient. Explanation of the columns is to be found in the text on pages 27 and 29.

						TIME OF DAY	1
						TIME INTERVAL IN SECONDS	2
						TIME ELAPSED IN SECONDS	3
						OUTSIDE OF INNER HOLE A	4
						OUTSIDE OF MINISCUS B	5
						MAXIMUM OF COMPONENT C	6
						INSIDE OF OUTER HOLE D	7
						REFERENCE DISTANCE H = D-A cm.	8
						INNER HOLE E_1 = B-A cm.	9
						OUTER HOLE E_0 = D-B cm.	10
						INNER HOLE X_1' = C-A cm.	11
						OUTER HOLE X_2' = D-C cm.	12
						$X_1'' = \frac{X_1'}{F}$ cm.	13
						$X_2'' = \frac{X_2'}{F}$ cm.	14
						$X_1 = 5.7 + f_1 + X_1''$ cm.	15
						$X_2 = 7.3 + f_2 - X_2''$ cm.	16
						$X = \frac{X_1 + X_2}{2}$ cm.	17
						Log X	18

MICROCOMPARATOR RDG. IN CM.

MINISCUS DISTANCE FROM

COMPONENT DISTANCE FROM

S-4720-8/64

$$f_1 = 0.02 \times \frac{\text{rpms} - 30,000}{59,780 - 30,000} \quad (3)$$

$$f_2 = f_1 \times \frac{5.7}{7.3} \quad (4)$$

In a cell rotating at 59,780 r.p.m., which is the maximum rotational speed of an analytical centrifuge, the stretching factors, f_1 and f_2 equal 0.02 and 0.0156 respectively (7). The values 5.7 and 7.3 are the actual distances in centimeters between the axis of rotation and the points A and D respectively. These are brought into the calculations in columns 15 and 16 of the chart. The logarithms in column 18 are graphed on the ordinate, while the values in column 3 are on the abscissa. The slope of the resultant line is divided by the square of angular velocity. This value is the observed sedimentation coefficient or s_{obs}. A correction factor for the temperature of the rotor and the density of the solution is multiplied by the s_{obs} to obtain the $s_{20,w}$ which is the sedimentation coefficient corrected to 20° C with water as a solvent. This factor multiplied by the s_{obs} equals the $s_{20,w}$ (7):

$$s_{20,w} = s_{obs} \frac{(\eta_t)\quad(\eta)\quad(1 - \bar{V}\rho_{20,w})}{(\eta_{20})\quad(\eta_o)\quad(1 - \bar{V}\rho_t)} \quad (5)$$

where: η_t/η_{20} = correction corresponding to the viscosity of water at the temperature of the rotor and at 20°,

η / η_o = relative viscosity of the solvent to that of water,

$\rho_{20,w}/\rho_t$ = ratio of the densities of water at 20° and of the solvent at t.

It is assumed that $\bar{V}$, partial specific volume, in the reference (water) solvent is the same as the actual solvent used in the experiment.

The $s_{20,w}$ is a standard value and is the usual form of the sedimentation coefficient which is reported.

2. Band Centrifugation.

Band centrifugation, as a technique for the study of interacting macromolecular systems, was simultaneously introduced by two groups of workers in 1963 (20, 89). It is a method of determining the sedimentation and diffusion coefficients of a thin layer, or lamella, of macromolecules placed upon a denser miscible liquid, or bulk solution. This system differs from the system of boundary centrifugation described above in the following ways: usually the macromolecules under investigation are distributed equally in the centrifuge cell and, in the process of centrifugation, centrifugal force affects the separation between the solute and the solvent. In band centrifugation, however, a single, thin layer of macromolecules is stabilized in its position in the cell by the difference in density between itself and the bulk solution. The centrifugal force does not cause the separation of the bulk solution from the macromolecular layer, but only produces the stabilizing density gradient for this band.

a. General equation:

The first attempt to describe band centrifugation mathematically was made by Vinograd (89). To simplify the derivation of equation 6 for the sedimentation coefficient of the band, he assumed a gaussian distribution of the sedimenting band during both the layering and the

sedimentation steps. This distribution was maintained by density gradients which result from the diffusion between the lamella and the bulk solution of smaller molecules less affected by centrifugal force, and also by the sedimentation of these smaller molecules in the centrifuge.

The Vinograd equation applies to non-interacting systems (89):

$$\ln r_o = s\omega^2 t + \text{constant} \qquad (6)$$

where: r_o = radial distance to band center.

Vinograd presented a more theoretical approach to the determination of the sedimentation coefficient of the band in another paper (90). Due to lack of the necessary equipment, this second approach is not workable (90).

b. Enzyme-substrate systems:

Cohen and co-workers, working with enzyme-substrate systems, have also derived equations for the calculation of the sedimentation and diffusion coefficients of interacting systems (20, 21). According to Cohen's equations, band centrifugation of an active enzyme substrate complex necessitates the following experimental conditions (21):

1. The efficiency of the enzyme must be constant in time.
2. The enzyme must be saturated with substrate throughout the cell. The rotating cell contains, therefore, a sedimenting enzyme which is permanently bound, an active enzyme substrate complex.
3. The reverse reaction, product to substrate, does not take place to any significant degree.

Mathematically, the derivation of equation 7, mentioned on page 32, is simplified by the following assumptions (21):

1. The sedimentation and diffusion coefficients are constant in time.
2. Edge effects do not exist. In the two edge regions of a normal cell located at the miniscus and at the bottom of the cell, reflected molecules in their back diffusion oppose the majority of the molecules in the solution. On the other hand, in this system it is assumed that this is a solution with "infinite boundaries". For this assumption to be valid, the band distribution must be negligible in the two edge regions.
3. The band distribution is assumed to be approximately gaussian. This is not true in practice, however, in the edge regions.
4. Precision data are needed only for the area of maximum concentration in the sedimenting band.
5. Up to 0.2% decrease in molecular mobilities from one end of the cell to the other is acceptable.

Their relationship is (21):

$$\text{Ln } R'/R_o = s\ \omega^2\ (t - t_o) \qquad (7)$$

where: R' = mean initial radius,

R_o = mean radius for the product,

t_o = zero time.

To determine the diffusion coefficient within 10% this equation must be used in conjunction with equation 1. Knowledge is required of the concentration distribution of the band. However, if information only of

the sedimentation coefficient is required, a 3-5% precision can be obtained if the mean radius is assumed to be the maximum concentration of the band (21). Then equation 7 reduces to Vinograd's equation, listed in this thesis as equation 6 (89).

c. Centerpieces:

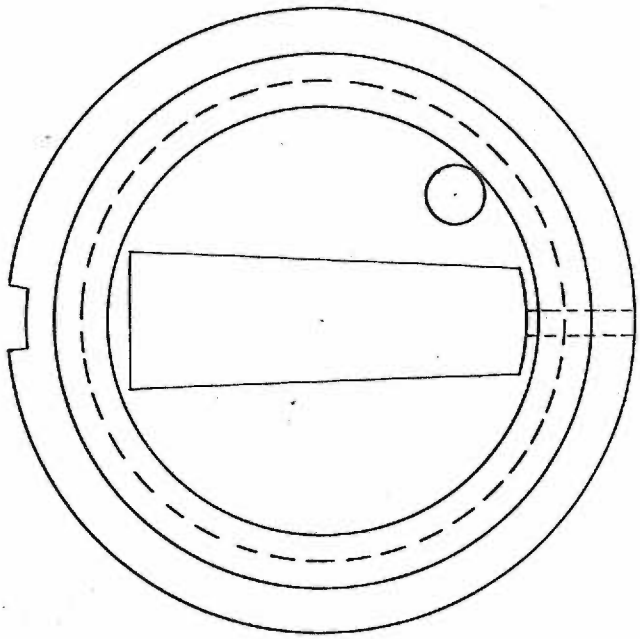
Several centerpieces have been designed for the purpose of transferring a given amount of solvent into the main sector of the centrifuge cell (8). This transfer occurs between 10,000 and 20,000 r.p.m.'s, at which speed the shear forces generated by the transfer cause denaturation of many proteins. For this reason, a more suitable centerpiece was designed by Vinograd to fulfill the requirements of band centrifugation (89). This centerpiece, described by Vinograd *et al.* (89, 92) and used in our experiments, is shown in Figure 3. It is a 12 mm Kel-F centerpiece with a 2.2 x 8 mm sample hole, which is 4.5 mm above and 4.4 mm lateral to the axis of the centerpiece and has a capacity of 30 microliters of sample solution. A raised bead around the circumference of the cell allows a 0.07 mm gap between the centerpiece and the quartz observation window and through this gap the sample is transferred during centrifugation. With this style of centerpiece, the quantity of bulk solution is limited to 0.55 ml. If more is used the amount of solvent displaced at the time of transfer causes continued contact with the sample hole, resulting in the appearance in the cell an hour or so after the start of centrifugation of artifactual bands which obscure the desired band (89, 92).

d. Band composition:

The constitution of the solution which is to form the thin

Figure 3:

This shows the diagram of the ultracentrifuge centerpiece used for our studies. It is described in the text on page 33. The inner circle indicates the minimum diameter of the raised bead. The dashed circle indicates the minimum diameter of the Kel-F cylinder. This cell was first described by Vinograd in reference 89.



layer, or lamella, is determined after consideration of the sedimentation and diffusion coefficients of the macromolecules in this solution. The optical systems which are used also influence lamellar concentration. For example, an absorption optical system is more sensitive than the schlieren optical system, so that more solute is required in a schlieren experiment. For practical purposes, however, the narrowest possible lamella, or band, is desirable, in order that the leading band edge will correspond to the point of maximum concentration. If the observed band is sharp on at least one side, the schlieren optical system may be practical, and the analyzer angle is set at 90° so that the light impinges upon the photographic plate having been deflected only by the solution through which it passes. The selection of experimental conditions is considered in reference 91.

e. Substrate solutions:

In active enzyme experiments, a small substrate molecule should be used which in itself does not affect the sedimentation coefficient of the complex. Monitoring the accumulation of a product or the disappearance of a substrate are preferred ways for observation of the enzymatic reaction within the sedimenting band (21). If neither product nor substrate has a specific absorption band, the enzymatic reaction must be coupled with another reaction so that it may be easily observed (21).

f. Recording data:

The primary experimental data, recorded on photographic plates, is utilized in either of two ways. The plate may be scanned by a densitometer to produce a graph of the density change (46). Alternatively, a

microcomparator of the type usually employed to read ultracentrifuge (31) plates will produce recordable data. Whether these data are obtained by densitometer or by microcomparator, after multiplication by the appropriate correction factor it is handled as described on pages 27 and 29.

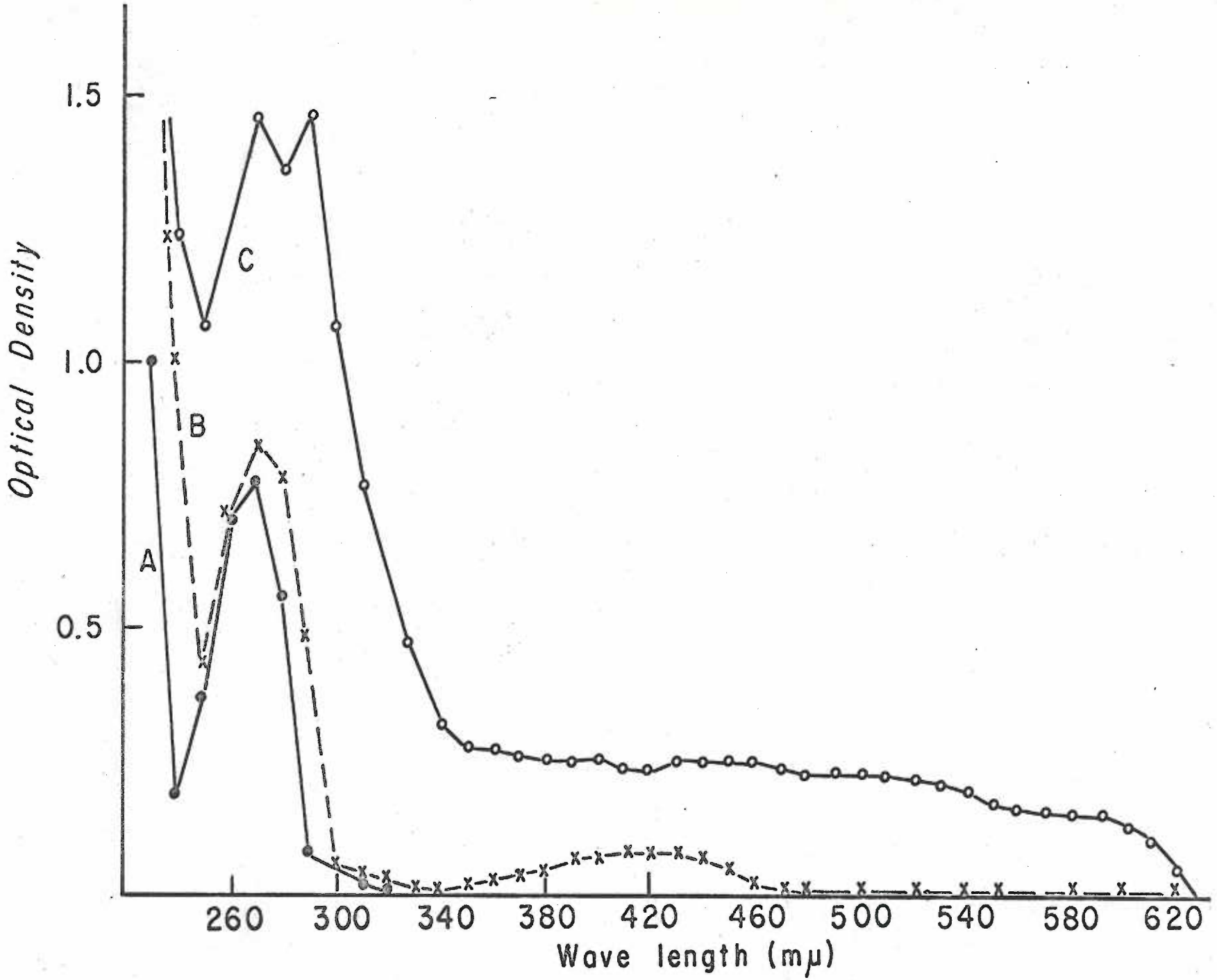
g. Experimental conditions:

In our experiments with the enzyme tyrosinase, the initial centrifugations were made with substrate of concentration 10^{-3} M. The buffer concentration was 0.01 M sodium phosphate or 0.01 M trishydroxylmethylaminomethane (tris) in 0.1 M NaCl. The choice of molarity was made on the basis of Cohen's suggestions (21). Buffers in the range of 0.05 to 0.01 M permit self generating density gradients to be obtained. These gradients are large enough to avoid convective disturbances during centrifugation, but sufficiently small so as not to retard the sedimenting molecules. The pH of our solutions was varied with buffers and runs were carried out at pH's 6.5, 6.9, and 7.4. From a time trace of the tyrosinase reaction (Figure 4) made with the Cary model 14 recording spectrophotometer, the absorption band of the initial product, 4-*tert*-butyl-o-quinone, was found to be at a maximum at 420 mμ. This product is measured spectrophotometrically since it represents the sedimentation of the tyrosinase-substrate complex. With a 420 mμ filter located in the light path, the data were recorded using blue sensitive I-O photographic plates (26).

In our later experiments the enzymatic reaction was coupled with the oxidation of nicotinamide adenine dinucleotide (NADH). The stochiometry of this reaction is (5, 54):

Figure 4:

Illustrated is the recording spectrum of a solution containing p-cresol buffer and enzyme, under the same conditions as used in the band centrifugation work with water as reference. The first curve, A, gives the spectrum of the solution of 10^{-3} M p-cresol, 0.01 M tris pH 7.4, and 0.15 N NaCl. Curve B shows the same solution immediately after adding 17 μg of γ enzyme. Curve C shows the original solution plus the added enzyme after 20 minutes.


1.5
1.0
0.5
Optical Density
A
B
C
260
300
340
380
420
460
500
540
580
620
Wave length (mμ)

$$\text{catechol} + 1/2\ O_2 \rightarrow \underline{\text{o}}\text{-quinone} + H_2O$$

$$\underline{\text{o}}\text{-quinone} + NADH_2 \rightarrow \text{catechol} + NAD^+$$

$$NADH_2 + 1/2\ O_2 \rightarrow NAD^+ + H_2O$$

NADH has an absorption maximum at 340 mμ. The concentration of NADH in the reaction mixture was calculated from its millimolar extinction coefficient at 340 mμ which is $6.22 \times 10^3\ cm^{-1}\ mM^{-1}$ (41). NADH concentration in these solutions was in the range of 10^{-3} M. A 340 mμ filter was used to observe the disappearance of NADH. Blue sensitive photographic plates I-O (26), were utilized as before. The plates recording the coupled reaction displayed a sharper band front than those of the previous experiments, and, thus, were more easily measurable. No change in the rate of sedimentation of the coupled reaction was observed. Since these plates were more easily interpreted, NADH was used in all subsequent experiments.

10-15 μg of tyrosinase were consumed in each repetition of the procedure. All the isozymes in turn were centrifuged in both substrates. The enzymes were diluted with 0.001-0.005 M phosphate buffer, pH 6.9, to a concentration of 1 mg per ml.

h. Experimental procedure:

See appendix.

D. GEL FILTRATION

1. General Introduction.

In 1959, Porath introduced Sephadex column chromatography as a rapid and inexpensive means of differentiating substances by their molecular weights (57). All means of molecular weight differentiation before this

time required expensive equipment and time consuming procedures. Sephadex consists of a cross-linked dextran in which the degree of cross linking determines the size of molecules which can be separated. From these Sephadex gel columns, the substances of greatest molecular weight are eluted first, followed by those of lesser molecular weight. This occurs because very small molecules such as buffer molecules can freely pass into the tiny pores of the gel beads. These smallest molecules, therefore, diffuse throughout the volume of the column and, in order of elution, they are the last to be eluted. Since larger molecules cannot enter into the gel particles, they pass through the column quickly and are eluted first. The fractionation range of a Sephadex gel includes that area of the molecular weight spectrum which can be differentiated by this material. All other molecular weights are said to lie in the exclusion limits of the gel and cannot be differentiated. Highly cross-linked gels such as Sephadex G-15 and G-25, have very narrow ranges of fractionation, while a relatively non-cross-linked gel such as G-200 may be used to separate all molecules except those of molecular weights less than 5,000 or greater than 800,000 (85).

2. Molecular Weight Determination.

Although Sephadex column chromatography is extensively used as a separation procedure, the potential of this method for determining molecular weights was not thoroughly investigated until 1964. In that year Andrews showed that the elution volumes of macromolecules could be directly correlated with their molecular weights if all the molecules under investigation had the same configuration (2). A column standardized with globular proteins, therefore, could only be used to

estimate the molecular weights of unknown globular proteins.

a. Selection of gel:

In our experiments, we wished to determine the relationship between the three aggregation states of the tyrosinase enzyme under different experimental conditions. The reported values for the molecular weight of the tyrosinase monomer are approximately 30,000, and those of the tetramer in the order of 120,000 (12, 45, 51, 81, 86); by interpolation, the dimer should have an approximate molecular weight of 60,000. These molecular weights are easily within the fractionation range of Sephadex gels from G-75 to G-200. While the fractionation range of G-200 is twice as broad as that of G-100, the gel is difficult to handle, its stability is short lived, and it allows only slow rates of flow during use. The technical properties of G-100 allow better operating conditions and the reported exclusion limits are 5,000 to 150,000 (3). Also, the linear portion of the separation curve of G-100 includes the molecular weights from 7,000 to 80,000 (3) which will differentiate both the monomeric and dimeric forms of tyrosinase. For these reasons, Sephadex G-100 was the material chosen for our experiments.

b. Column preparation and standardization:

Before each of our five separation procedures, the Sephadex G-100 was immersed and swollen for three days in 0.02 M phosphate pH 6.7 (85). The dimensions of the first column were 1.5 x 35.5 cm, but this column was used only for preliminary experiments and it was not standardized. The other four columns each had diameters of 2.5 cm and lengths ranging from 32 to 49 cm. Each column was standardized using proteins recommended by Andrews (3). In order of molecular weight, the

proteins used were: glucose oxidase, molecular weight 153 x 10^3; lactic dehydrogenase, 130-140 x 10^3; serum albumin, 65-70 x 10^3; maleic dehydrogenase, 55-70 x 10^3; peroxidase, 39-44 x 10^3; myoglobin, 17.8 x 10^3; and cytochrome c, 12.4 x 10^3 (2, 3, 38). The void volume of each column was determined with blue dextran, 10^6 in molecular weight. Four standardization experiments were done through each column, utilizing any four of the above standards per experiment. The location of each standard in the elution pattern was determined from its absorption concentration at 280 mμ whenever possible (3). If the column to be standardized had been previously equilibrated with 4-*tert*-butylcatechol, which also has an absorption pattern at 280 mμ, proteins which could be located by their enzymatic activity or which possessed absorption peaks other than at 280 mμ were chosen for the standardization experiments.

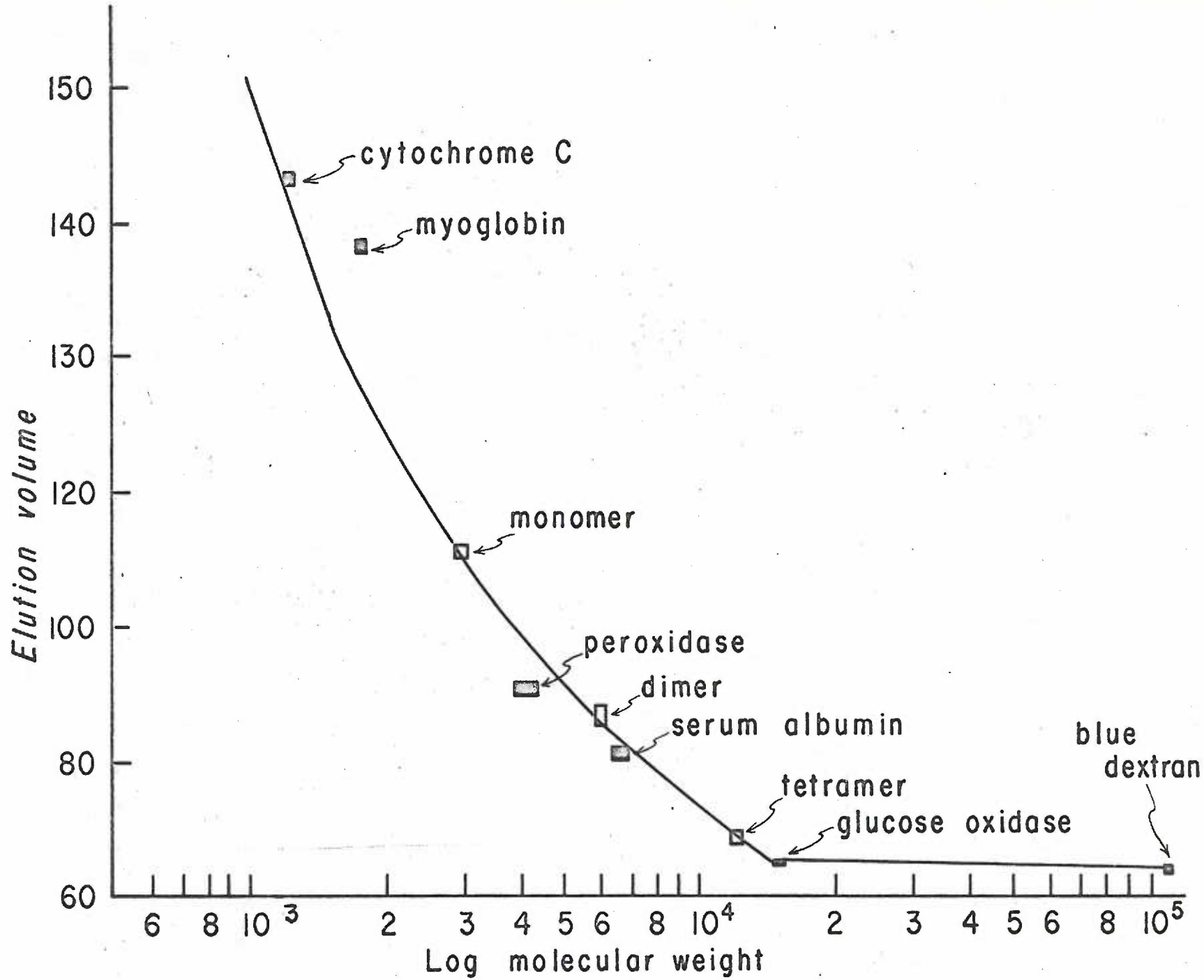
A semi-logarithmetic graph was produced which correlated the elution volumes, which represent the maximum concentrations of each component, to the known molecular weights of these compounds. The result was a graph of the range of elution volumes to be expected for each aggregation state of tyrosinase (3) (see Figure 5).

c. Experimental procedure:

One of the isozymes, *alpha*, *beta*, or *gamma*, was used for each column experiment. In a volume of 1 ml, applied to the column in each experiment, the amount of enzyme ranged from 3.0-500 μg. Some experiments were run on columns with no substrate, under both anaerobic and aerobic conditions; substrates used were 5 x 10^{-3} M 4-*tert*-butylcatechol, anaerobically and aerobically; 5 x 10^{-3} M 4-*tert*-butylphenol, anaerobically and aerobically; and 5 x 10^{-2} M and 5 x 10^{-4} M 4-*tert*-butylcatechol,

Figure 5:

Typical standardization curve for a Sephadex G-100 column. Column dimensions were 2.5 x 49 cm. The elution rate was 2.2 ml per 2 minutes, and the chromatography was carried out at room temperature. Fractions were collected every two minutes.


150
140
130
120
100
80
60
Elution volume
cytochrome C
myoglobin
monomer
peroxidase
dimer
serum albumin
tetramer
glucose oxidase
blue
dextran
6 8 10 3 2 3 4 5 6 8 10 4 2 3 4 5 6 8 10 5
Log molecular weight

aerobically.

d. Aerobic experiments:

For the aerobic experiments, a standard Pharmacia column apparatus was used (80). Sephadex gel was poured into this column mold and allowed to settle overnight before undertaking the standardization. Each column employed gravity flow with buffer reservoirs positioned one foot above the level of the top of the column. The sample was applied by interrupting buffer flow and allowing the sample to flow onto the column by gravity, and then reconnecting the buffer reservoir. All column runs were conducted at room temperature with flow rates averaging 1 ml per minute. Samples were collected in a Buchler fraction collector (16), both in aerobic and in anaerobic experiments. Collection commenced with the onset of the sample application.

e. Anaerobic experiments:

Our anaerobic column apparatus was patterned after the design by Munson and co-workers (67) (see Figure 6). This apparatus, although adaptable to anaerobic collection of samples, was not employed for this purpose in our experiments[2].

The Sephadex bed was poured aerobically and the resulting column was then connected to the apparatus. After assembly of the apparatus and filling of the pyrogallol trap and of the buffer chamber, hydrogen was flushed through the system overnight to render the buffer solution and the apparatus anaerobic. Prepurified hydrogen was further deoxygenated by a deoxy-catalytic filter (32), connected directly to the tank, and by placement of a vanadyl sulfate trap in the hydrogen system ahead of the

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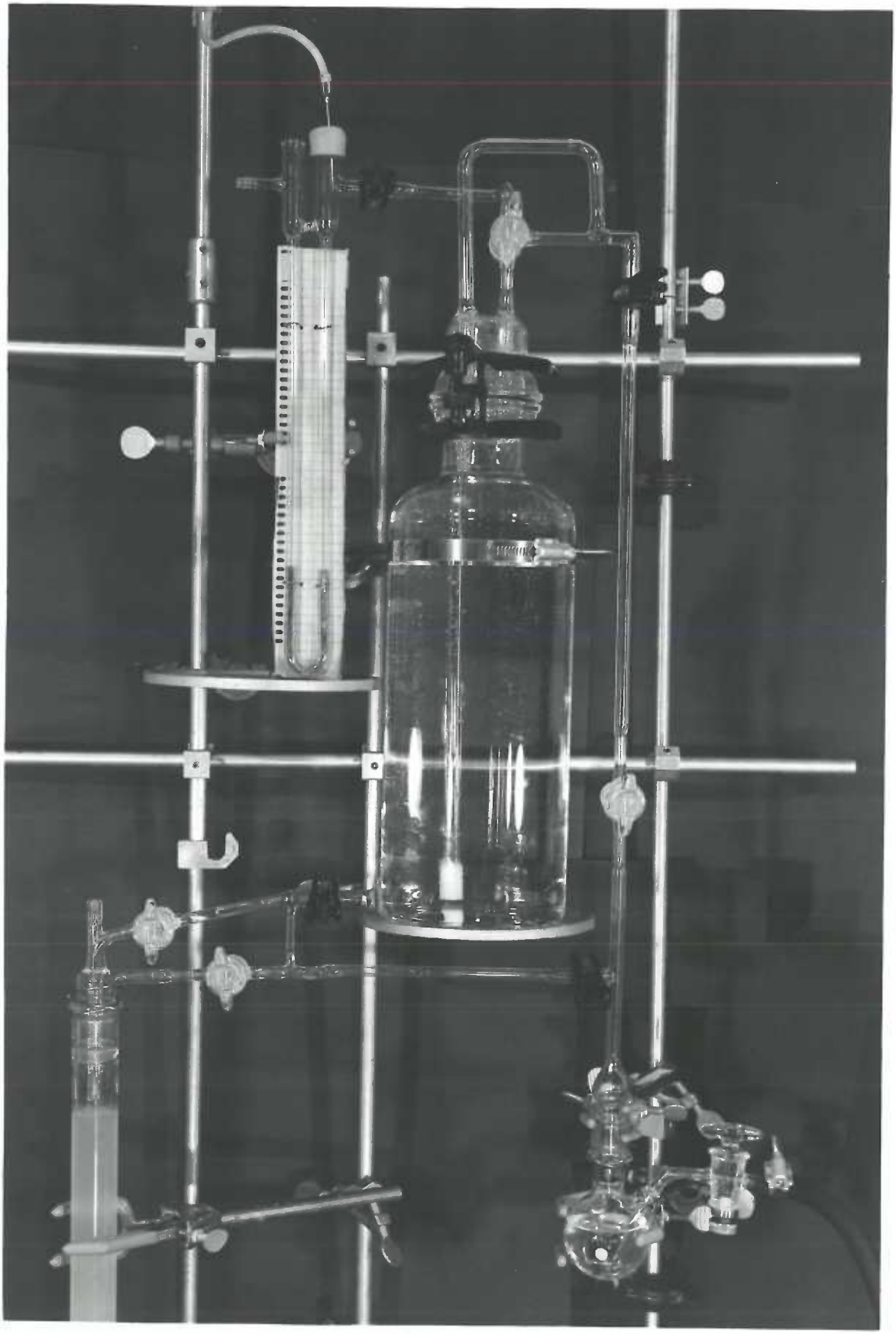
2. The assistance of Mr. Gunther Weiss in the design and production of this equipment is gratefully acknowledged.

Figure 6:

The anaerobic column apparatus. In the lower right corner is the trap containing 50 ml of pyrogallol solution. In the center is the buffer reservoir which holds 3.5 liters of buffer. A manometer in the upper left corner records the pressure of hydrogen gas in the apparatus, when the needle is removed from the serum cap. The lower left corner shows the top of the column bed. Directly above the column is a rubber plug through which the sample is placed on the column. The apparatus is all glass with O-ring connectors. The only non-glass portion of the system, after the pyrogallol trap, is the rubber plug for sample application.



pyrogallol trap. Potassium hydroxide solution for the pyrogallol trap was vacuum deoxygenated and flushed with prepurified nitrogen. This procedure was repeated several times to insure complete oxygen removal before addition of this solution to the trap. After ten minutes of flushing with hydrogen, the trap was disconnected from the apparatus and a weighed amount of solid pyrogallic acid was added to the potassium hydroxide solution. The pyrogallol solution was made up of 50 g of potassium hydroxide and 2.5 g of pyrogallic acid which is combined with 50 ml of water (39). The apparatus was then reassembled as quickly as possible. This procedure was considered to result in an adequately anaerobic system because, although an initial brown hue was imparted to the pyrogallol solution from its brief contact with air, over the period of a week this color was not observed to darken significantly. The Sephadex gel column was made anaerobic by washing with a volume fifteen times the capacity of the column (67).

If substrate was to be added to the buffer solution, the buffer in the chamber was first deoxygenated as described above. Then a weighed amount of substrate was added to the chamber, and this solution was flushed with hydrogen for a minimum of two hours. The addition of substrate necessitated exposure of the apparatus to the air, so that this procedure was carried out as rapidly as possible.

Before application to the column, each sample was diluted with distilled water to a volume of 1 ml, in a test tube. The tube was then sealed with a serum stopper and fitted with a stopcock. These sample containing tubes were then evacuated and flushed with prepurified nitrogen. Excess solvent was allowed to drain from the column, and

then, with a syringe and a very fine needle, the sample was applied dropwise directly onto the top of the Sephadex column. This procedure proved to give a sharper band front than rapid injection of the sample onto the bed. When the sample had been completely absorbed into the gel, the buffer chamber was opened, and the substrate solution allowed to spread over the surface of the column. Fractions were collected in volumes of 2.0-2.2 ml per tube and the collection was begun at the instant of sample application (16).

f. Elutant assay system:

In assaying the elutant from the columns, both those developed under anaerobic and those from aerobic conditions, one of two procedures was followed depending upon the equilibrating solution which was used in the column.

To tubes containing solution from columns without substrate, 0.1 ml of 6 mM 4-*tert*-butylcatechol was added, and the color, which was monitored at 420 mμ, was allowed to develop for 30 minutes. Those from columns equilibrated with substrate were already quite colored. To each of these tubes an appropriate dilution of 5×10^{-3} M ascorbic acid was added until the solution just became colorless. The volume added was always less than five drops in order to minimize the optical density change due to dilution. The color was then allowed to develop 30 minutes and then recorded at 420 mμ. The equilibrating buffer which was used with all columns was 0.02 M phosphate, pH 6.7.

g. Linearity of assay system:

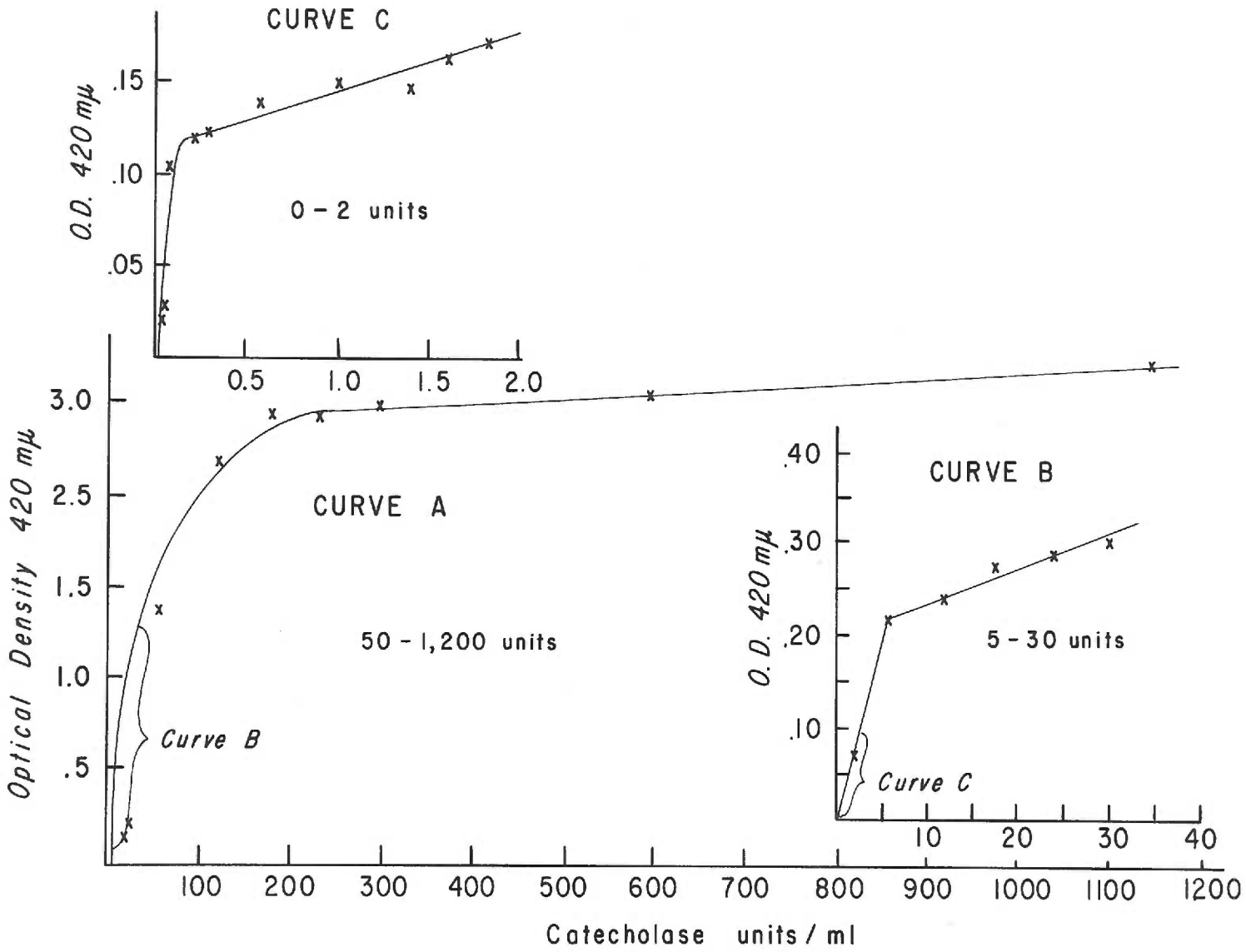
We sought to establish if a linear relationship existed between the optical density at 420 mμ and the enzyme concentration in our

ascorbic acid procedure for activity assay of the elutant from the columns. Using enzyme concentrations ranging from 0.02 µg/ml to 546 µg/ml, an experiment was designed to evaluate the curve. Each portion of *gamma* enzyme, diluted to 0.9 ml with water, was mixed with 0.1 ml of a 5×10^{-2} M solution of 4-*tert*-butylcatechol. After allowing the enzyme to react for one hour, five drops of a dilution of 5×10^{-3} M ascorbic acid was added to each tube, until the solution became colorless. Then the color was allowed to develop for an additional thirty minutes and recorded following this interval. This procedure duplicated as closely as possible the conditions encountered in a column experiment.

Although the assay method was found not to be linear over the entire range of concentrations in the experiment, within limited ranges, such as from 0-500 nanograms per ml (0-2 catecholase units), from 2-150 µg/ml (5-30 catecholase units), and from 50-450 µg/ml (100-1,200 catecholase units), the method gave fairly linear results with respect to enzyme concentration (see Figure 7). It should be noted that, with an average difference of 40 ml between volumes of elution of the monomer and the tetramer, the initial linear portion of this curve would include all those columns to which were applied samples of 0-10 µg. The second linear range includes those samples between 10-100 µg applied concentration, and the third, those from 100-500 µg applied concentration. Although the optical density reading at 420 mµ is not absolutely proportional to enzyme concentration, within a range of concentrations the highest optical density reading of any tube corresponds to the greatest amount of enzyme. Also, it should be noted that just 6 nanograms of enzyme per ml (0.007 catecholase units) elicits a change in optical

Figure 7:

This graph illustrates the degree of linearity between enzyme concentration and the optical density reading in the ascorbic acid assay system, and the relationships between each portion. Curve A, the major curve, covers the ranges from 50-1,200 catecholase units (50-450 µg/ml), curve B covers the range below 50 units, from 5-30 catecholase units (2-15.0 µg/ml), and curve C the range below 5 units, from 0-2 catecholase units (0-500 nanograms/ml).


CURVE C
O.D. 420 mμ
.15
.10
.05
0 - 2 units
0.5
1.0
1.5
2.0
Optical Density 420 mμ
3.0
2.5
1.5
1.0
.5
CURVE A
50 - 1,200 units
Curve B
CURVE B
.40
.30
.20
.10
O.D. 420 mμ
5 - 30 units
Curve C
10
20
30
40
100
200
300
400
500
600
700
800
900
1000
1100
1200
Catecholase units / ml

density from the baseline value. These results indicate that this is a very sensitive assay system, and that if the peak is not recorded, it amounts to less than 0.006 micrograms of enzyme.

The column experiments were carried out twice for each set of conditions.

E. ASSAY TECHNIQUES

The catecholase activity was determined by the chromometric method of Dawson (23). Cresolase activity was determined manometrically, according to the procedure of Greg and Nelson (36). The manometric flasks used in this procedure contained 3.4 ml of fluid with a p-cresol concentration of 1 mg/ml (36). To facilitate the monitoring of the fractions eluted from the hydroxylapatite columns, a modified method for determining activity was employed (44, 72). The rate of oxygen consumption catalyzed by the enzyme was measured by a Clark-type O_2 electrode (8), attached to a recorder. The electrode was fitted into a flat bottomed cell, water jacketed for a constant temperature of 25°. Three ml of substrate solution containing 1 mg/ml 4-*tert*-butylcatechol (6 mM) in 0.1 M sodium phosphate-0.05 M citrate buffer, pH 5.1, were added to the cell and stirred with a magnetic stirrer. An appropriate amount of enzyme solution (1-50 µl) was then added to the cell. A unit was defined in terms of µmoles O_2/minute and was calculated on the basis of 1 ml of added enzyme solution. The activities measured in this manner are shown to be proportional to the enzyme concentration up to an oxygen consumption of 0.36 µmoles/min/3 ml substrate solution (44, 72).

Final protein concentrations were obtained by the Folin's method

(55). To estimate the protein concentration as eluted from hydroxylapatite columns, the optical density of each tube was read at 280 mμ.

Copper content was determined directly from the enzyme in the presence of 2,2'-biquinoline in glacial acetic acid (27). Ascorbic acid reduced the cupric copper to cuprous for assay. Under these conditions the molar extinction coefficient was 6.8×10^3 mM^{-1} cm^{-1} at 540 mμ (27).

F. CHEMICALS

Catechol and p-cresol were redistilled from Eastman Kodak chemicals practical grade. 4-*tert*-butylcatechol was purified from an Aldrich Chemical, practical grade product either by petroleum ether extraction or by sublimation. The melting point of the 4-*tert*-butylcatechol used in our experiments was 54-55°. 4-*tert*-butylphenol was sublimated from a Matheson, Coleman, Bell product to a melting point of 99-100°. Literature value for this compound was 99° (24). The 3,4-dimethylphenol used in these experiments was reagent grade produced by Eastman Kodak. The NADH was a product of Sigma Chemical Company, Grade III. All phosphate buffers were sodium salts. The proteins used for standardization were Sigma products, best commercial grade. All other chemicals used were of the best commercial grade available. All solutions were made with deionized distilled water.

Hydroxylapatite was obtained from Bio-Rad Laboratories and was prepared by the Tiesulius method (87). The Sephadex G-25 and G-100 were swollen in the equilibrating buffers for the length of time specified by Pharmacia (80, 85). Columns made from these materials were

poured in one operation and allowed to settle overnight before use (85).

Ultracentrifuge plates used for the band centrifugation work were I-O plates, blue sensitive, from Eastman Kodak (26). The plates used in the schlieren work were metallographic plates, green sensitive, also an Eastman Kodak product (26).

A Zeiss spectrophotometer with a 1 cm light path was used to record all optical densities from the column chromatography (96).

RESULTS

A. PURIFICATION

The purification scheme outlined above is the best thus far devised for the preparation of mushroom tyrosinase. Because it does not require complex equipment, it can be followed by any investigator. The lead subacetate precipitation is the most difficult step, but if utmost care is taken, even this step may present no problems. All other steps are quick, dependable, and repeatable. The degree of purity of the final product is comparable to that reported by other workers who used more elaborate procedures (12, 51). Although this method separates the enzyme into its four isozymes, nevertheless, these are consistently pure and homogeneous as tested by ultracentrifugation and by acrylimide gel electrophoresis. This scheme has proved for us to be extremely useful.

B. ULTRACENTRIFUGATION STUDIES

The centrifugation results depicted in Figures 8 through 11 were obtained in order to analyze the homogeneity of each concentrated fraction. In order to correlate these results with the sedimentation results reported by other investigators, a series of ultracentrifugation experiments were attempted to show the variation of the sedimentation coefficient with the concentration of the enzyme. Extrapolating the s°_{20} value obtained in the above experiments to infinite dilution, we obtained a s°_{20} value of 7.15 S for β-tyrosinase in 0.02 M phosphate buffer (see Figure 11). Similar experiments on *alpha*, *gamma*, and *delta* enzymes in 0.026 M borate buffer gave values of 6.62, 7.10, and 7.29

Figure 8:

The sedimentation pattern of β, upper, and α, lower, tyrosinase. The concentrations of α and β were 10.0 and 4.91 mg/ml respectively. The rotor speed was 52,640 r.p.m.'s. Pictures were taken at 16 minute intervals. Sedimentation is from bottom to top.

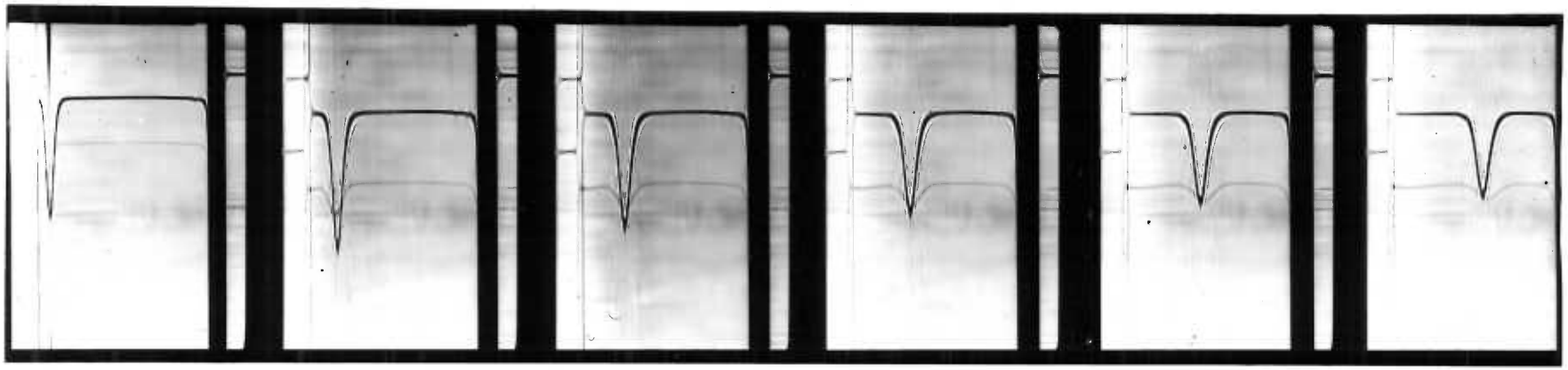


Figure 9:

The sedimentation of γ-tyrosinase in the presence (lower) and the absence (upper) of substrate. Details of the centrifugation are similar to those given in Figure 8 except that the centrifugation temperature was 25° C. The enzyme concentration was 5.4 mg/ml and the 3,4-dimethylphenol concentration was 10^{-3}. Sedimentation was from bottom to top.

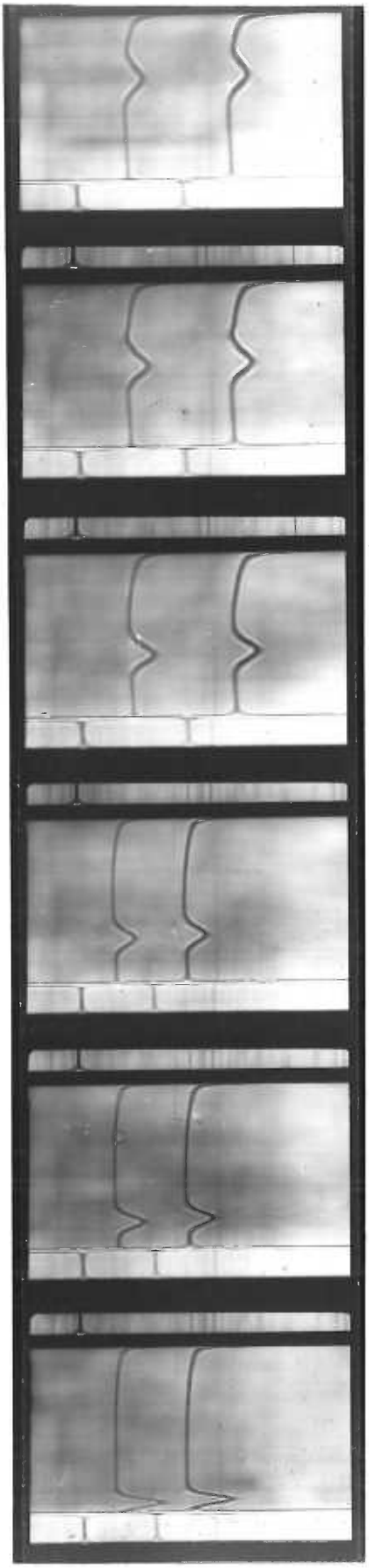


Figure 10:

The δ enzyme before (upper) and after (lower) chromatography on Sephadex G-100. Concentrations were 9.8 and 5.7 mg/ml (upper and lower, respectively). Conditions of the centrifugation are the same as Figure 9; pictures were taken as in Figure 8. Sedimentation was from bottom to top.

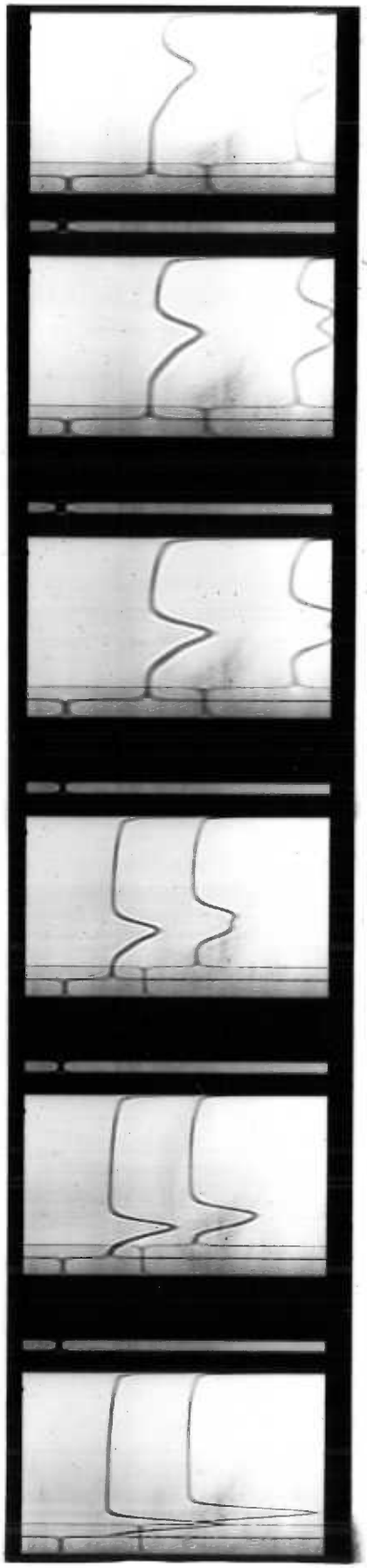
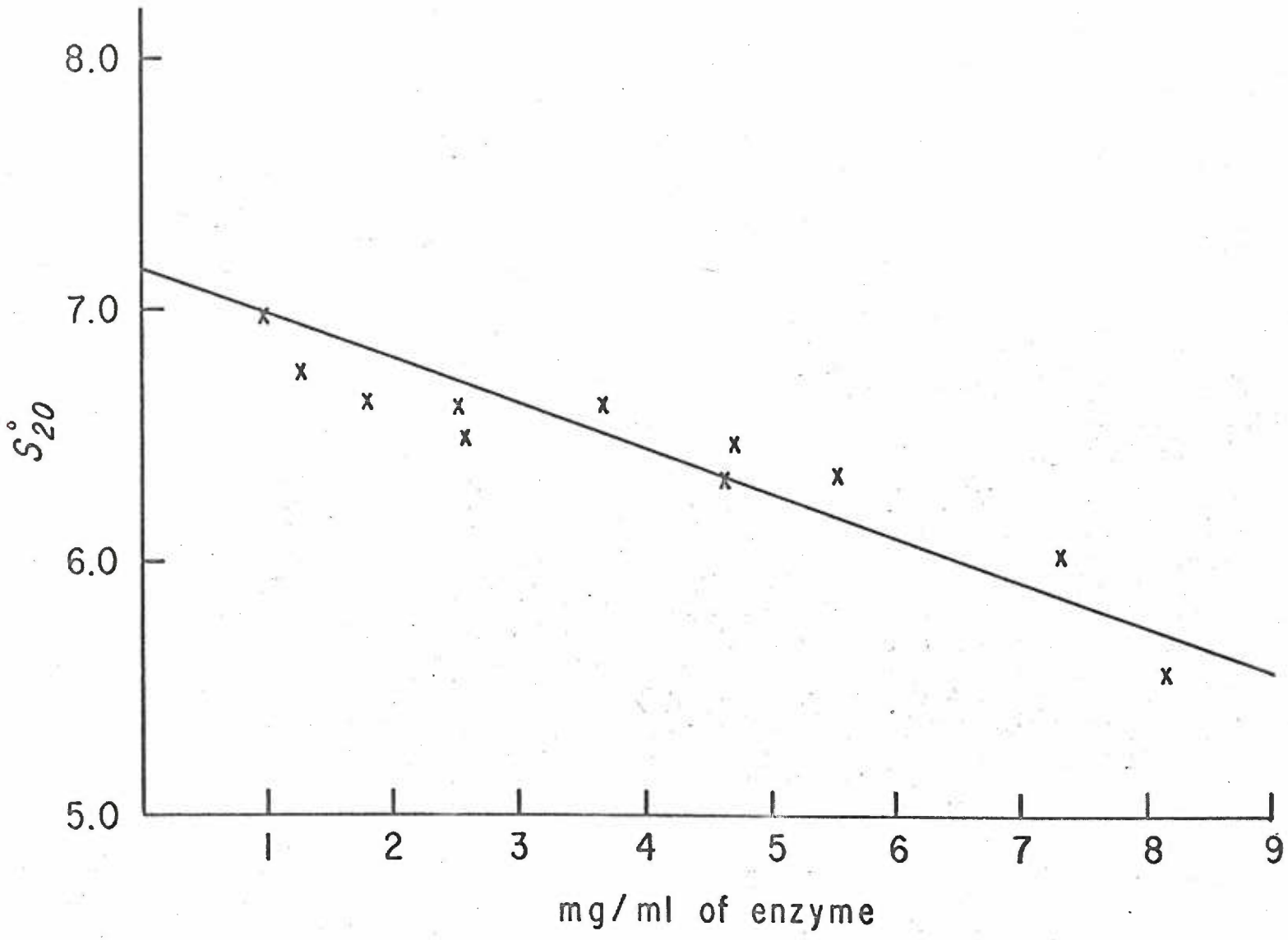


Figure 11:

The change in the sedimentation constant of β-tyrosinase upon dilution of this enzyme. The rotor temperature was kept at 20° throughout the sedimentation period, and the buffer concentration was 0.02 M phosphate pH 6.5.


8.0
7.0
6.0
5.0
S°_{20}
1
2
3
4
5
6
7
8
9
mg/ml of enzyme

S respectively (45).

C. BAND CENTRIFUGATION

1. Summary of Results.

Band centrifugation sedimentation coefficients of tyrosinase were 2.51 ± 0.73 S in the presence of monophenol (25 experiments) and 2.30 ± 0.18 S for the diphenol-enzyme complex (22 experiments). The temperature in these experiments ranged between 20-25° C. All the isozymes of tyrosinase were used to obtain these averaged values (see Table II).

Samples of the two typical patterns to be expected from a tyrosinase band centrifugation experiment are provided in Figure 12. The calculation necessary to obtain the s_{obs} from these figures is also provided. One of the patterns was scanned with a chromoscan (46) and the other was measured with a microcomparator (31). The data were treated as described in the Methods section, pages 27-29 of this thesis.

The ultracentrifuge, a Beckman model E (8), used for these experiments lacks a unit for direct temperature control of the rotor while in the chamber, although the chamber is refrigerated during the centrifugation. A temperature check on the rotor is obtained before starting an experiment and again after the experiment is completed. These two readings are averaged to give the temperature of the experiment. For this reason, the rotor should be removed from its chamber and its temperature evaluated as soon as possible after the experiment is completed to insure the accuracy of the temperature reading.

This centrifuge is equipped with a schlieren optical system, but is

Table II:

This table lists the results obtained with each isozyme which were averaged to give the results reported in the text. The substrate used is listed, and the number of experiments which were averaged to give the reported figures also included.

TABLE II

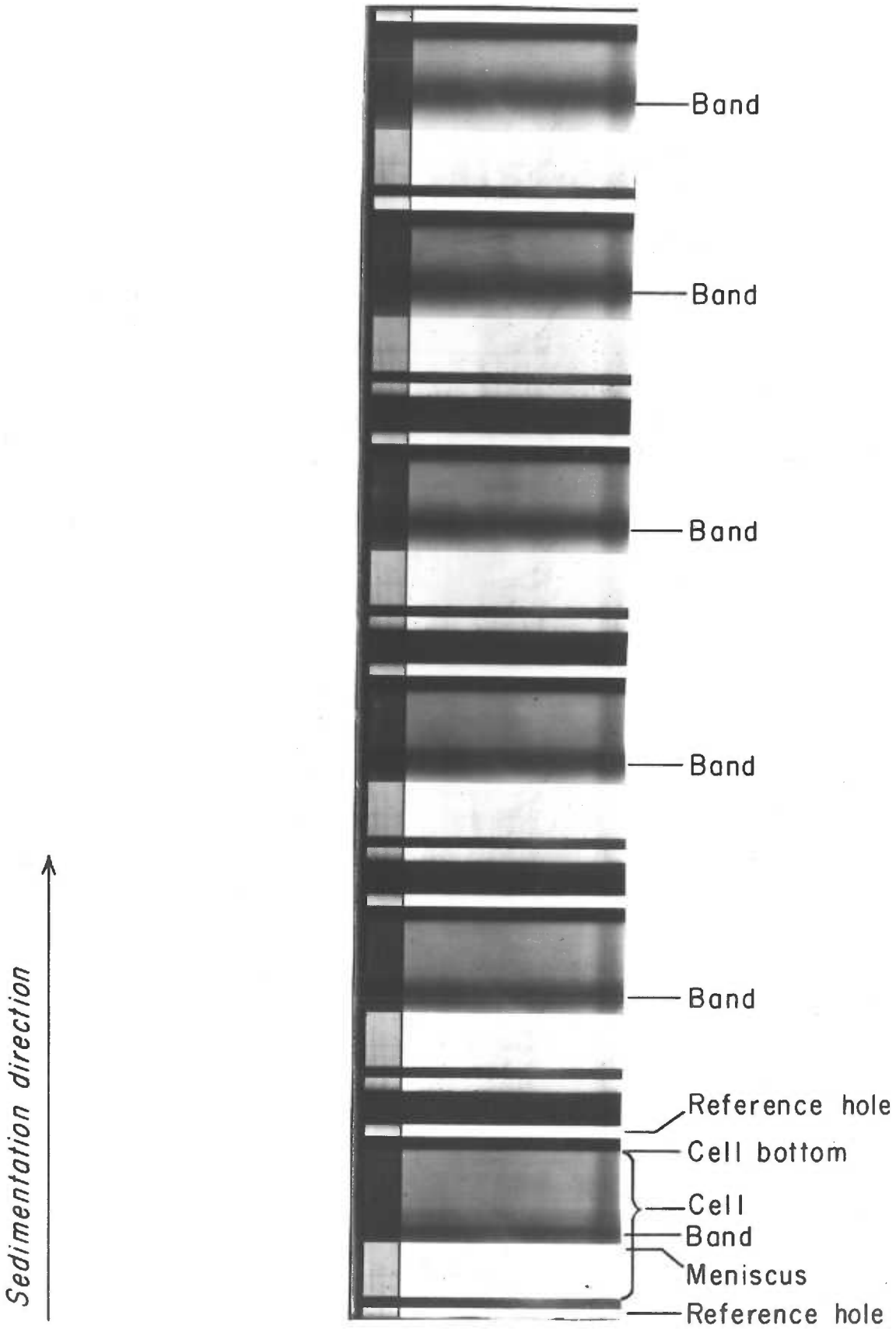
Isozyme	Monophenolic Substrate		Diphenolic Substrate
	p-cresol	tyrosine	catechol
alpha	-	2.81 (4)*	2.17 (5)
beta	-	2.91 (4)	2.35 (5)
gamma	2.55 (5)	2.41 (7)	2.30 (9)
delta	-	2.57 (4)	2.44 (3)

*Parenthesis denote number of experiments performed with the isozyme in the presence of the named substrate. Values are averages of the experiments.

Figure 12:

A comparison of the pattern obtained with NADH in the substrate solution (A) and without NADH (B).

(A) Experimental conditions are: 9 µg of δ-tyrosinase layered onto a solution containing 0.1 M NaCl, 10^{-3} M catechol, and 2.3×10^{-3} M NADH in 0.01 M phosphate buffer, pH 6.4. Pictures were taken at 16 minute intervals and the sedimentation followed with a 340 mµ filter. (See calculations for experiment I-19) The enzyme band is viewed as a dark area which travels from bottom to top in the illustration.


Band
Band
Band
Band
Band
Reference hole
Cell bottom
Cell
Band
Meniscus
Reference hole
Sedimentation direction

A. Experiment I-19:

Rotor speed = 59,780 r.p.m.

340 mμ filter.

F = 21.0.

f_1 = .020.

f_2 = .015.

TIME INTERVAL IN MINUTES	18. Log X
0	.81451
16	.82014
32	.82458
48	.82627
64	.82898
80	.83378

The above numbers are graphed on the succeeding page, labeled experiment I-19, and the s value calculated.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
TIME OF DAY	TIME INTERVAL IN SECONDS/MINUTES	TIME ELAPSED IN SECONDS	MICROCOMPARATOR RDG. IN CM.				REFERENCE DISTANCE H = D-A cm.	MINISCUS DISTANCE FROM		COMPONENT DISTANCE FROM							
			A OUTSIDE OF INNER HOLE	B OUTSIDE OF MINISCUS	C MAXIMUM OF COMPONENT	D INSIDE OF OUTER HOLE		INNER HOLE E_1 = B-A cm.	OUTER HOLE E_0 = D-B cm.	INNER HOLE X_1' = C-A cm.	OUTER HOLE X_2' = D-C cm.	$'' X_1 = \frac{X_1'}{F}$ cm.	$'' X_2 = \frac{X_2'}{F}$ cm.	$X_1 = 5.7 + f_1 + X_1''$ cm.	$X_2 = 7.3 + f_2 - X_2''$ cm.	$X = \frac{X_1 + X_2}{2}$ cm.	Log X
11:47	0		5.196	19.391	22.245	39.242	34.046			17.049	17.049	.8119	.8119	6.547	6.500	6.524	
12:03	16		6.314	21.012	25.375	40.836	34.522			19.061	15.461	.9077	.7362	6.643	6.576	6.609	
12:19	32		9.565	24.276	30.029	44.113	34.548			20.464	14.084	.9745	.6707	6.710	6.641	6.677	
12:35	48		11.419	25.115	31.459	44.970	34.551			21.040	13.511	1.0019	.6434	6.737	6.669	6.703	
12:51	64		15.368	30.037	37.192	49.904	34.536			21.825	12.712	1.0393	.6053	6.784	6.707	6.745	
1:07	80		7.333	22.628	30.743	41.893	34.560			23.410	11.150	1.1148	.5310	6.860	6.781	6.820	

S-4720-8/64

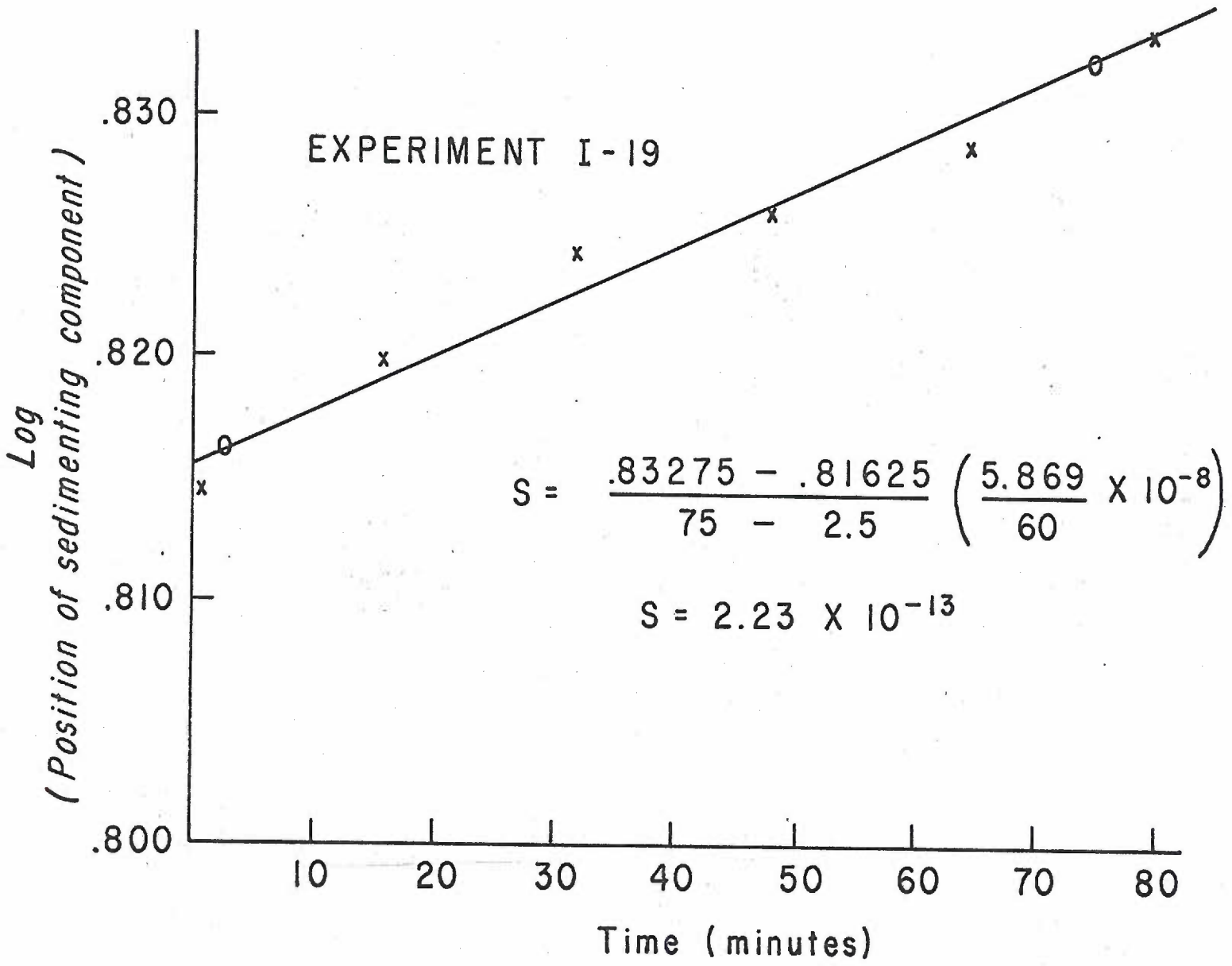
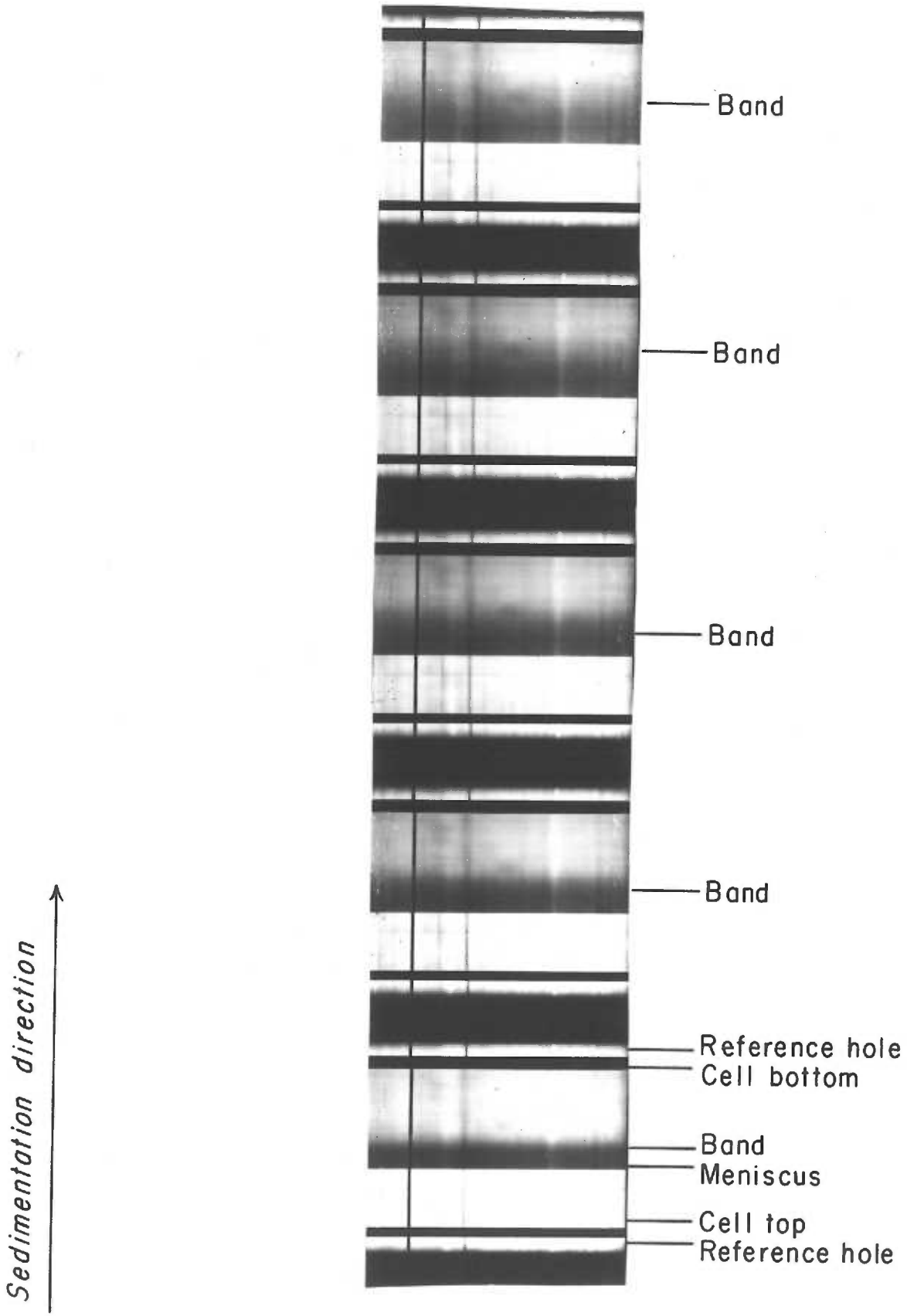

EXPERIMENT I-19
Log
(Position of sedimenting component)
.830
.820
.810
.800
10
20
30
40
50
60
70
80
Time (minutes)
$S = \frac{.83275 - .81625}{75 - 2.5} \left(\frac{5.869}{60} \times 10^{-8} \right)$
$S = 2.23 \times 10^{-13}$

Figure 12:

(B) Experimental conditions are: 10 μg of γ-tyrosinase layered onto a solution containing 0.1 M NaCl and 10^{-3} M p-cresol in 0.01 M tris pH 7.4. Pictures were taken at 8 minute intervals and the plates scanned with the chromoscan. The sedimentation was followed with a 420 mμ filter (see calculations for experiment 513). The enzyme band, in this illustration, is viewed also as a darker area traveling from bottom to top, but which now does not separate from the meniscus leaving a clear area behind it, but simply becomes more diffuse as it sediments. The chromoscan graphs of this centrifuge plate are included following page 62.


Band
Band
Band
Band
Reference hole
Cell bottom
Band
Meniscus
Cell top
Reference hole
Sedimentation direction

B. Experiment #513:

Rotor speed = 52,640.

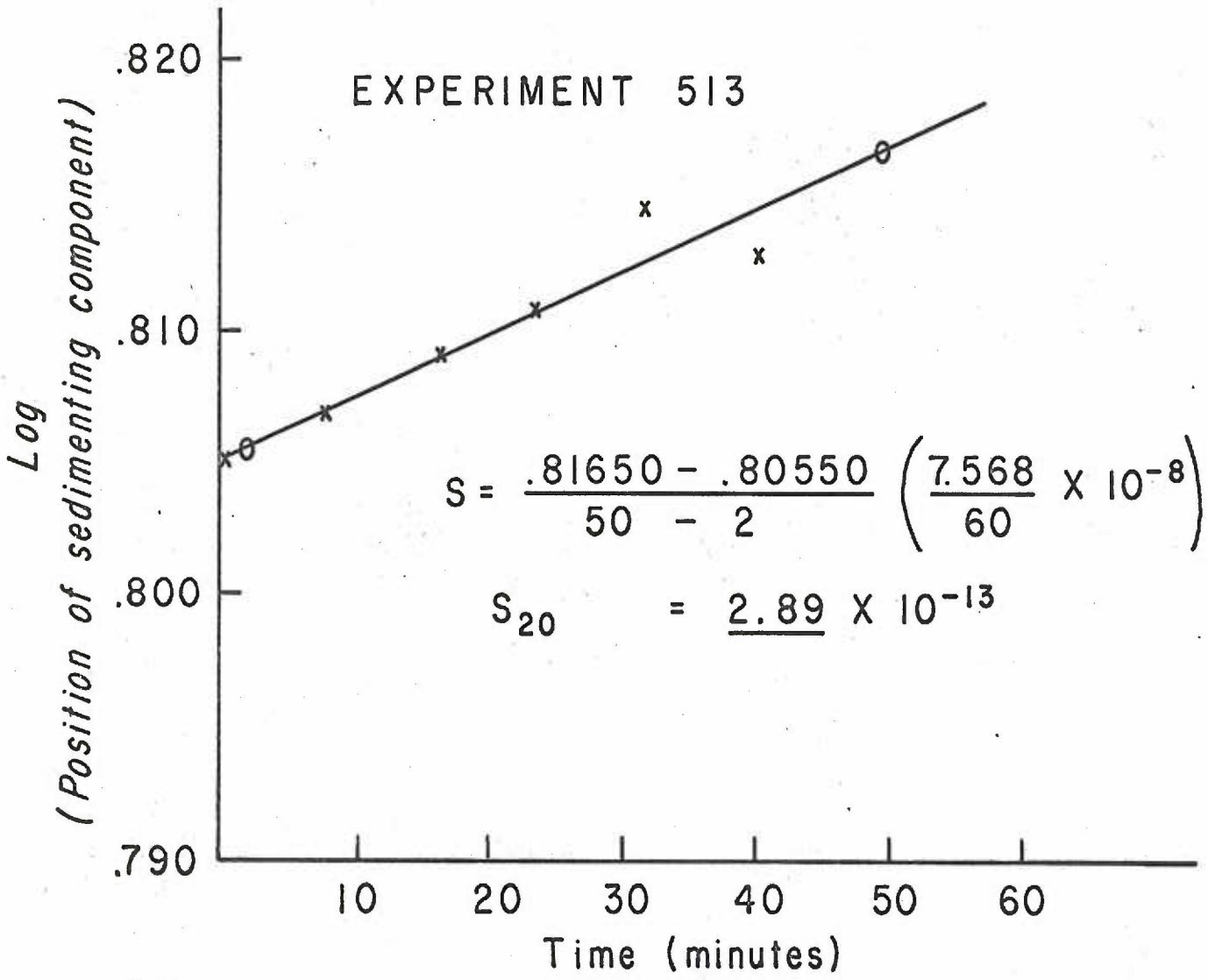
Temperature = 20°.

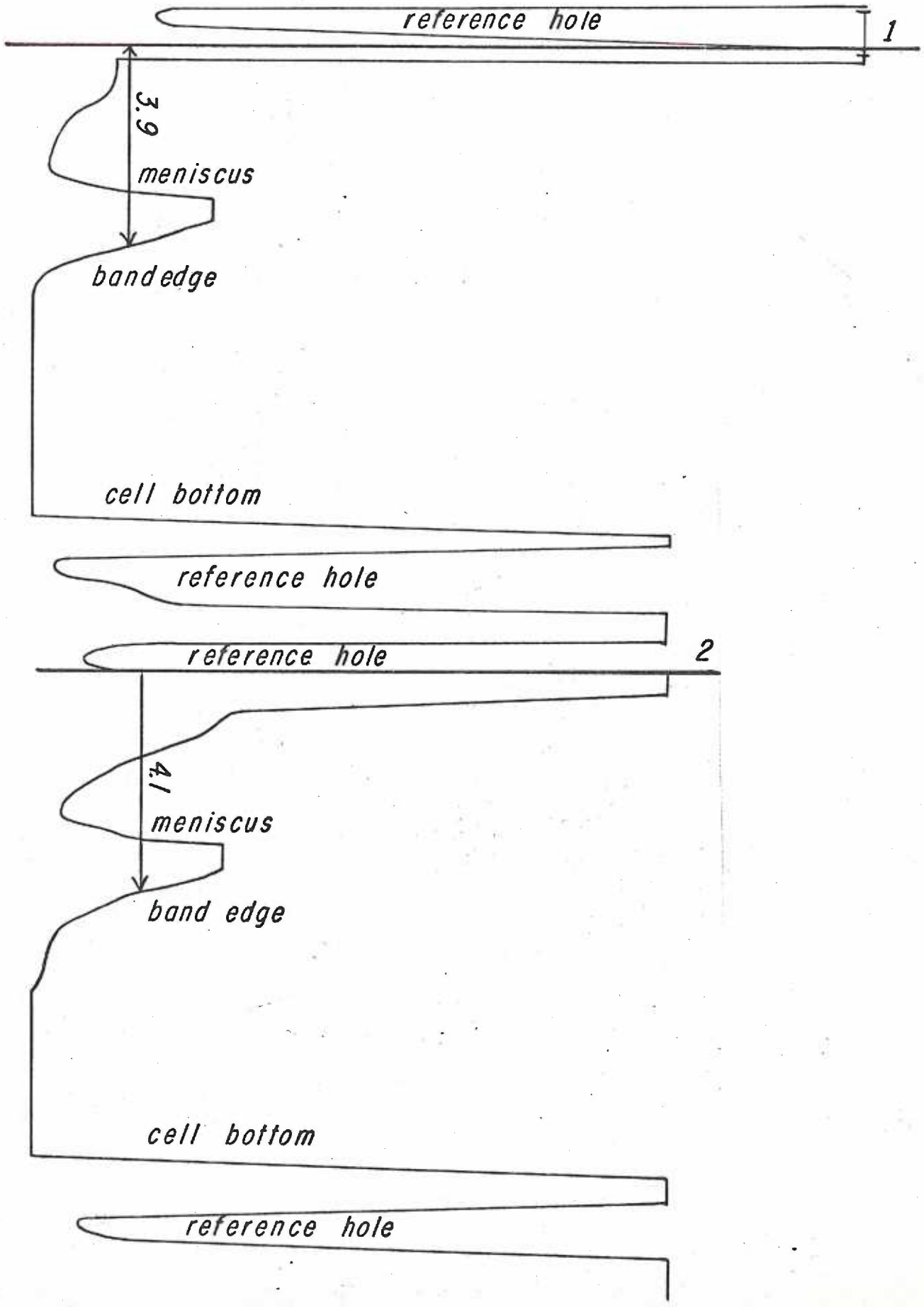
420 mμ filter.

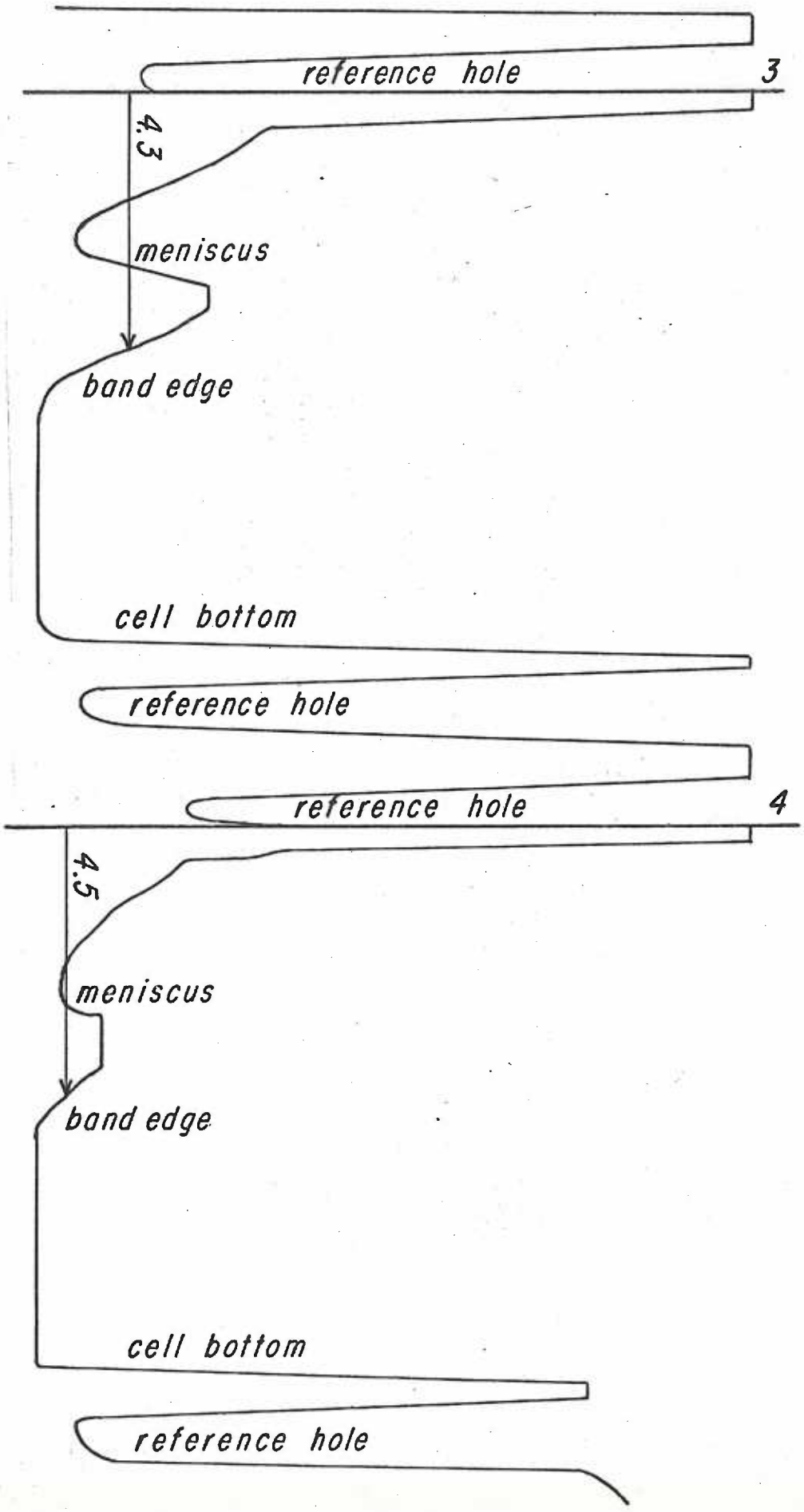
TIME INTERVAL IN MINUTES	18. Log X
0	.79671
8	.79865
16	.80058
24	.80250
32	.80632
40	.80441

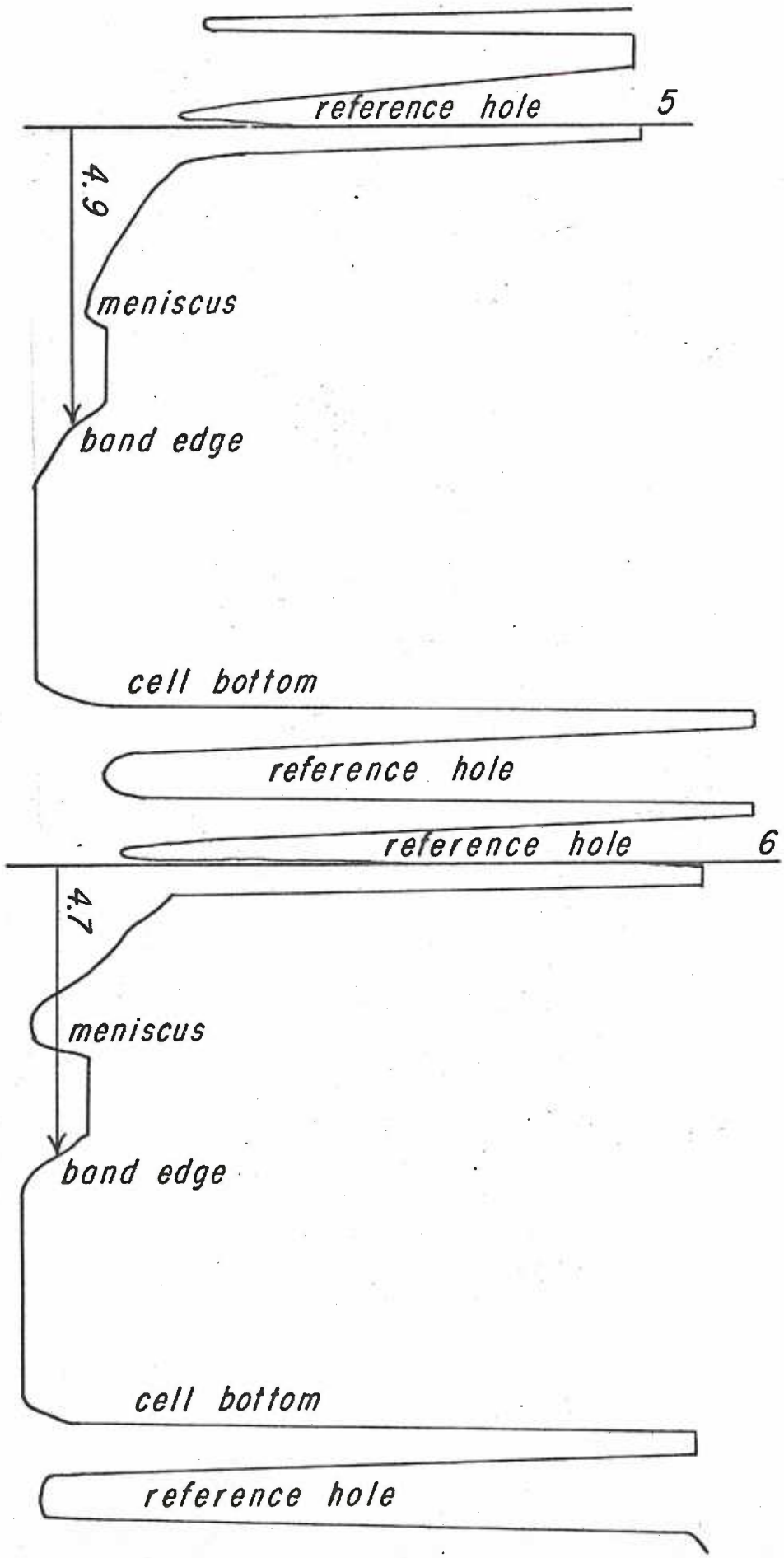
The above numbers are graphed on the succeeding page, labeled experiment 513 and the S values calculated.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
TIME OF DAY	TIME INTERVAL IN SECONDS MINUTES	TIME ELAPSED IN SECONDS	MICROCOMPARATOR RDG. IN CM.				REFERENCE DISTANCE H = D-A cm.	MINISCUS DISTANCE FROM		COMPONENT DISTANCE FROM		$X''_1 = \frac{X_1}{F}$ cm. $X^1 \cdot (.1391)$	$X''_2 = \frac{X'_2}{F}$ cm.	$X_1 = 5.7 + f_1 + X''_1$ cm.	$X_2 = 7.3 + f_2 - X''_2$ cm.	$X = \frac{X_1 + X_2}{2}$ cm.	Log X
			A OUTSIDE OF INNER HOLE	B OUTSIDE OF MINISCUS	C MAXIMUM OF COMPONENT	D INSIDE OF OUTER HOLE		INNER HOLE E_1 = B-A cm.	OUTER HOLE E_0 = D-B cm.	INNER HOLE X'_1 = C-A cm.	OUTER HOLE X'_2 = D-C cm.						
4:40	0				3.9				3.9			.542		6.262			
4:48	8				4.1				4.1			.570		6.290			
4:56	16				4.3				4.3			.598		6.318			
5:04	24				4.5				4.5			.625		6.346			
5:12	32				4.9				4.9			.682		6.402			
5:20	40				4.7				4.7			.654		6.374			


EXPERIMENT 513
Log
(Position of sedimenting component)
.820
.810
.800
.790
10
20
30
40
50
60
Time (minutes)
$S = \frac{.81650 - .80550}{50 - 2} \left(\frac{7.568}{60} \times 10^{-8} \right)$
$S_{20} = \underline{2.89} \times 10^{-13}$


reference hole
1
3.9
meniscus
band edge
cell bottom
reference hole
reference hole
2
4.1
meniscus
band edge
cell bottom
reference hole


reference hole
3
4.3
meniscus
band edge
cell bottom
reference hole
reference hole
4
4.5
meniscus
band edge
cell bottom
reference hole


reference hole
5
4.9
meniscus
bond edge
cell bottom
reference hole
reference hole
6
4.7
meniscus
bond edge
cell bottom
reference hole

without an absorption system.

Preliminary work with tyrosinase and band centrifugation was done by Dr. Russell L. Jolley, Jr., of our laboratory. Substrate, 10^{-3} M 4-*tert*-butylcatechol in 0.1 M NaCl and 0.01 M tris pH 7.4, was the original buffered solution. The observed sedimentation coefficient for the tyrosinase enzyme substrate complex, under these conditions was 2.20 S^3. In this same series of experiments, when p-cresol was used as substrate the results could not be interpreted. The observed band varied from a sedimentation value of 30 to one of 60 S. Analysis of the experimental procedure revealed that 0.75 ml of substrate solution had been added to the centerpiece rather than the suggested volume of 0.55 (89, 92) and the bands obtained were apparently artifacts resulting from the use of too much substrate solution in the center sector of the ultracentrifuge cell (89, 92).

2. Control Experiments.

Two control experiments were performed: one duplicating all the experimental conditions in the absence of enzyme, the other with layered enzyme but no substrate in the ultracentrifuge cell. No band was observed in either case, nor in experiments with boiled enzyme.

3. Varying Experimental Conditions.

The majority of our experiments were done with the solution as described previously in the Methods section (pages 36-38) of this thesis. The effects of varying the sedimentation conditions were also studied. The following results were obtained.

It has been observed that high ionic strength causes the dissociation of tyrosinase (42, 43). In one series of our experiments, therefore, the sodium chloride concentration in the substrate solution was varied from the standard 0.1 M to 0.2 M NaCl[3] (Table III). As these changes in ionic strength were found not to have any significant effect on the computed sedimentation coefficient, and since the reference solution conventionally used in ultracentrifuge experiments is 0.1 M NaCl, we felt that this salt concentration was optimum for use in the majority of our experiments.

Because the o-quinone produced by the enzymatic reaction polymerizes to form melanin (59), which obscures the initial reaction product, it was at first thought best to retard the reaction in the initial steps. It was most feasible to vary the oxygen concentration, so that this was made the rate limiting factor. This involved deoxygenating all substrate solutions under vacuum and flushing these solutions with helium. Each solution was treated in this manner four times before final equilibration with prepurified nitrogen. The operations of preparing the ultracentrifuge cell for an experiment were done in a glove bag in a nitrogen atmosphere (Table IV). The sedimentation value obtained after this procedure was not significantly altered by a limited oxygen concentration, and although the interpretability of the developed plates was improved, the degree of this improvement was not judged to be proportional to the effort involved in the above procedures and the

---

3. For these initial experiments the cooperation of Wilbur Engel, a graduate student at Oregon State University, is gratefully acknowledged.

Table III:

This table illustrates the effect of increasing salt concentration on the sedimentation coefficient of the γ isozyme in the presence of a p-cresol concentration of 10^{-3} M. The buffer used for all these experiments was 0.01 N tris pH 7.4.

TABLE III

Isozyme	Salt Concentration*		
	0.1 M	0.15 M	0.2 M
gamma	2.54	2.30	2.49

*p-cresol was the substrate used in all these experiments, and its concentration was 10^{-3} M.

Table IV:

This table illustrates the results obtained when the oxygen concentration in the sedimentation solution was limited. The γ isozyme was used in these experiments in the presence of both p-cresol and tyrosine.

TABLE IV

Isozyme	Substrate	N_2*	No N_2
gamma	p-cresol	2.73	2.55
gamma	tyrosine	2.73	2.41

*s_{20} values.

deoxygenation step was discontinued.

Although variation of pH, ionic strength, buffer species, or oxygen concentration had little effect on the observed sedimentation coefficient, nevertheless the effect of a change in substrate concentration needed to be studied. Experiments were designed over a hundred fold range of substrate concentrations from 10^{-2} to 10^{-4} M (Table V). It is possible that 10^{-4} M substrate does not fulfill the experimental conditions initially prescribed by Cohen *et al.* (21), that the substrate must remain saturating throughout the cell. The average of the sedimentation coefficients obtained in these experiments with catechol as substrate was 2.47, which is very close to the values reported for our other work.

It is felt that the experiments outlined above explore the range of variables significant to this method of reaction analysis, and that the sedimentation coefficients reported here are correct. These are 2.51 ± 0.73 S for the monophenol, and 2.30 ± 0.18 S for diphenol.

4. Other Sedimentation Results.

Treating β-tyrosinase with succinic anhydride, or studying the sedimentation pattern of the same isozyme in the presence of 10^{-3} M ethylene diamine tetracetic acid (EDTA), produced a species which has a sedimentation coefficient at 20° of between 4.1 and 4.4 S. Upon addition of calcium to the enzyme solution containing EDTA, the original enzyme was regained. The values for a parallel run with concentrated, non-treated enzyme in these experiments were 6.65 and 6.68 S (44). As the coefficient values of 4.3, 4.4, and 4.12 S are considerably greater than those produced by band centrifugation, methods to confirm the band

Table V:

The change in sedimentation coefficient of the *beta*-isozyme with a change in the substrate, catechol, concentration over a hundred-fold range. The graphs of the position of the sedimenting component, given as the log of this value, plotted against the time, are also included (Figure 13, A and B). To be noted in particular, is the rapid inactivation of the *beta* isozyme in the presence of 10^{-2} M catechol as is observed by the change in the slope of the curve, and also the apparent back sedimentation of this band.

TABLE V

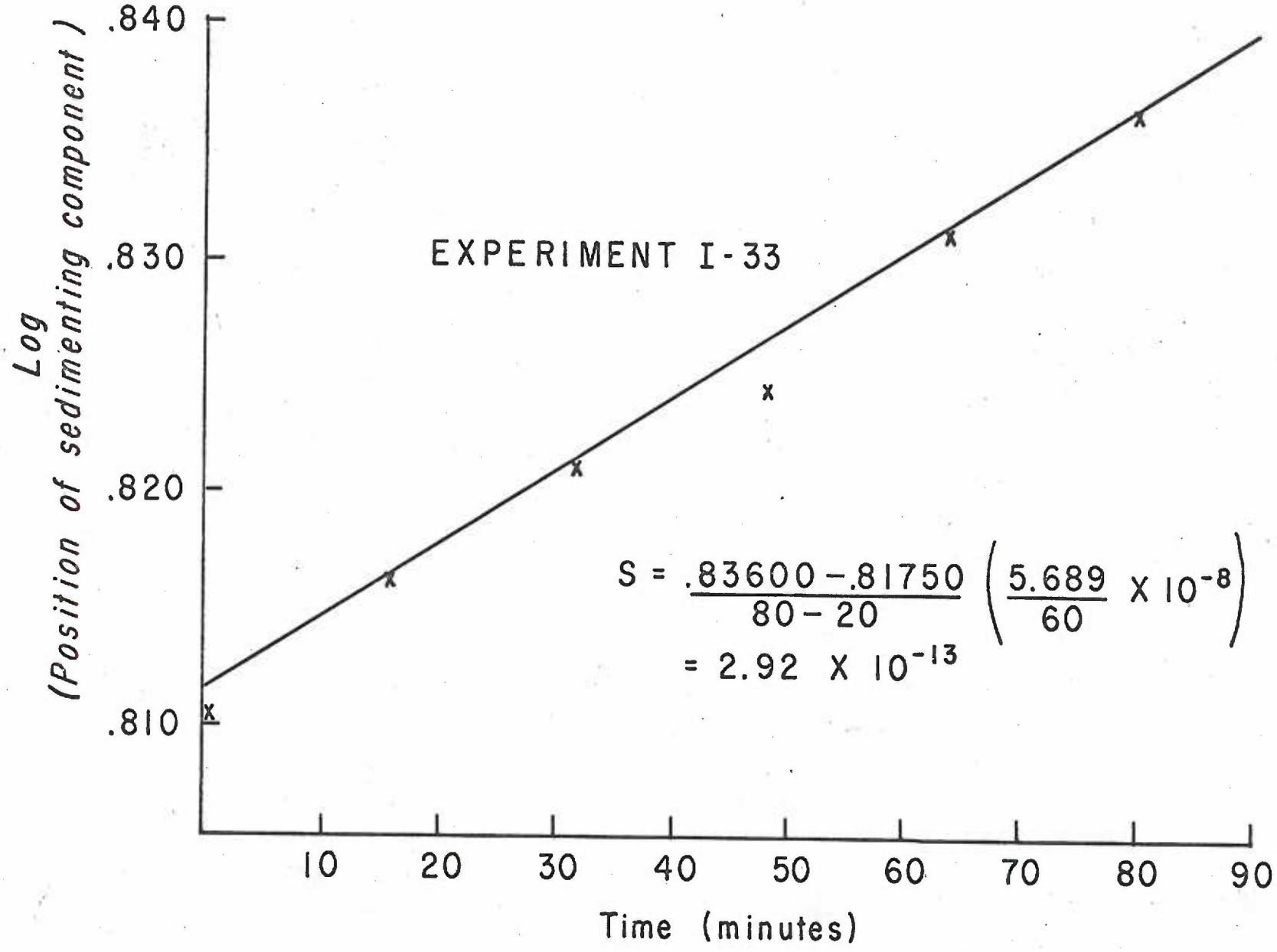
Isozyme	Substrate Concentration 10^{-2} M	10^{-3} M	10^{-4} M
beta	2.09*	2.42	2.92#

*enzyme inactivated quickly.

#calculations included, experiment I-33.

Figure 13:

This figure illustrates the position of the sedimenting component with respect to time. The results obtained from this graph is summarized in Table V. The experimental conditions are 13 μg of β enzyme layered onto a solution containing 10^{-4} M catechol and 10^{-4} M NADH. The rotor speed in this experiment was 59,780 r.p.m.


EXPERIMENT I-33
Log
(Position of sedimenting component)
.840
.830
.820
.810
10
20
30
40
50
60
70
80
90
Time (minutes)
$S = \frac{.83600 - .81750}{80 - 20} \left(\frac{5.689}{60} \times 10^{-8} \right)$
$= 2.92 \times 10^{-13}$

centrifugation work were devised[4].

A boundary centrifugation of enzyme in the presence of substrate was attempted. 3,4-dimethylphenol was chosen as substrate because its reaction product does not form polymers, so that the o-quinone product may be dialyzed to recover the pure enzyme (71). When this suggestion was followed no effect on the sedimentation pattern of γ-tyrosinase was observed, and the sedimentation coefficients were found to be 7.05 S in the presence of 3,4-dimethylphenol, and 7.18 S in its absence (see Figure 9).

D. GEL FILTRATION RESULTS

1. Preliminary Results.

Gel filtration was explored as an alternate method of determining the state of aggregation in the enzymatic reaction (49, 94). In preliminary experiments, a Sephadex G-100 column was equilibrated with 10^{-3} M p-cresol and 0.04 M phosphate buffer at pH 6.7, and 100-500 micrograms of enzyme were then applied to the column. These amounts of enzyme in a concentration of 1 mg/ml were used in all the preliminary experiments. The elutant showed one color peak, corresponding to the void volume of the column. The condensation products produced from enzymatic reaction with p-cresol obscured the position representing the protein and made interpretation of the data difficult. Because of the small quantities of enzyme present, measurements of the peaks for activity could not be made. Therefore, for the purpose of data inter-

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4. This work was done in conjunction with Dr. Russell L. Jolley, Jr.

pretation the locus of maximum concentration of the colored product was assumed to be the position of the enzyme. Another peak of color at approximately twice the void volume of the column was noted. This peak may have been the elution volume of the column for the p-cresol polymerization product.

To facilitate interpretation of our results, we decided to use 4-*tert*-butylcatechol as substrate. This diphenol is an excellent substrate for tyrosinase, as its o-quinone does not polymerize to form melanin-like products (70). Reaction inactivation does not occur when the enzyme reacts with 4-*tert*-butylcatechol as substrate, as occurs with other catecholase substrates (70). We chose 10^{-3} M substrate concentration (in 0.02 M phosphate buffer at pH 6.7) to insure that enzyme would be saturated with substrate throughout its passage through the column. The molarity of the buffer is that suggested by Pharmacia to minimize any interactions between the gel support and the applied protein (85). In two experiments with the same range of enzyme concentrations as before the elution speed was varied from 1 ml per minute to 1 ml per six minutes. After all fractions were collected, ascorbic acid was added to the individual collection tubes as described in Methods. A two-peaked pattern resulted from recording the optical density of these tubes at 420 mμ. The first peak was located at the void volume of the column, but the second peak was at 1 2/5 times the void volume and was repeatable in both experiments. The distance between these peaks seemed to be independent of the rapidity of elution, but their integrity was enhanced by an increase in elution speed.

In another of our preliminary experiments, ascorbic acid in a concentration of 5×10^{-3} M was added to the substrate solution of 10^{-3} M

4-*tert*-butylcatechol, and the column was equilibrated with this same buffer. The elution pattern was recorded and no difference in the position or number of peaks was observed with this column from those developed with substrate alone. Since meaningful results were being obtained, another, larger column was prepared and standardized as explained in Methods.

2. Control Chromatography.

When the *beta* isozyme of tyrosinase was applied at a concentration of 6.86 µg/ml to the standardized Sephadex column equilibrated with 0.02 M phosphate, only the peak corresponding to the tetramer was observed. If the applied concentration was in the range of 100-500 µg per ml, a three-peaked elution pattern was observed corresponding to tetramer, dimer, and monomer. The dilute *gamma* enzyme (17.2 µg/ml, buffered in an anaerobic environment) showed a two-peaked pattern with a small dimer peak and a major monomer peak. If the applied concentration of this isozyme was increased to the 100-500 µg/ml range, a two-peaked pattern was still produced. These peaks appeared in approximately equal ratios, but still represented only dimer and monomer, and no tetrameric peak was seen (Table VI).

In contrast to these findings with *beta* and *gamma* enzymes, the *alpha* isozyme, when applied in amounts less than 10 µg/ml could not be located by our detection method. If the applied concentration was 37.5 µg/ml, only the tetrameric peak was eluted. Two peaks were in evidence when the concentration was in the range of 100-500 µg/ml, representing the tetramer and dimer. In the anaerobic columns without substrate, no

Table VI:

This table shows the results of the columns equilibrated with 0.02 M phosphate, pH 6.7, both the anaerobic and aerobic columns. Details are in the text, pages 73 and 75.

TABLE VI

Isozyme	Concentration Applied (µg)	Recovery (%)	Tetramer Amount	Tetramer %	Dimer Amount	Dimer %	Monomer Amount	Monomer %
			AEROBIC					
beta	500.	93	254.	51	171.	34	42.	8
	171.5	89	58.9	34	54.9	32	38.8	23
	6.9	92	6.4	92				
alpha	500.	94	254.	51	217.	43		
	150.	88	79.	53	52.8	35		
	37.5	90	33.8	90				
			ANAEROBIC					
gamma	510.	89	-		220.	43	238.	46
	17.2	87	-		4.0	23	11.	64
alpha	375.	89	-		162.	43	171.	46
	37.5	91	-		15.2	41	18.9	50

alpha monomer was observed. From the anaerobic column with 37.5 µg applied concentration, only the monomer peak was observed. More concentrated *alpha* produced similar results to those of concentrated *gamma*. As with the *gamma* isozyme, no tetrameric peak was evident on the anaerobic columns. These results are summarized in Table VI.

3. Chromatography in the Presence of Diphenol.

The results from the aerobic columns equilibrated with 5×10^{-3} M 4-*tert*-butylcatechol are shown in Table VII. The appearance of the monomer in the very dilute enzyme applications is to be noted. It seems that as the concentrations of the *alpha* and *beta* enzymes decrease, the amplitude of monomer peak in proportion to those of the dimeric and tetrameric peaks increases. The anaerobic Sephadex chromatography showed essentially the same pattern as that noted in the absence of substrate, as only monomer and dimeric peaks were evident. The only major discrepancy between these anaerobic columns and those not equilibrated with substrate was that a dimeric peak was not seen with dilute *alpha* enzyme preparations in the columns equilibrated with substrate.

One column, which was equilibrated with 5×10^{-2} M 4-*tert*-butylcatechol and developed with 3.4 µg of *beta* enzyme, eluted three peaks corresponding to all three aggregation states. However, the greater concentration of 4-*tert*-butylcatechol and the increased activity of the enzyme in this preparation colored the column with quinoid products which we were not able to remove with repeated washings. No repetition of this experiment, therefore, was attempted.

More dilute substrate, 5×10^{-4} M 4-*tert*-butylcatechol, was also

Table VII:

This table summarizes the results from the columns equilibrated with 5×10^{-3} M 4-*tert*-butylcatechol in 0.02 M phosphate, pH 6.7.

TABLE VII

Isozyme	Concentration Applied (µg)	Recovery (%)	Tetramer Amount	Tetramer %	Dimer Amount	Dimer %	Monomer Amount	Monomer %
			AEROBIC					
beta	500.	91	168.4	34	284.	57	-	-
	172.	88	31.7	18	98.2	57	22.7	13
	34.3	83	3.75	11	21.6	63	3.16	9
	17.0	86	3.84	22	8.9	52	2.07	12
	3.4	85	0.66	19	1.0	30	1.21	36
alpha	375.	90	249.	66	47.5	13	42.	11
	30.	86	6.6	22	8.8	29	10.7	35
	15.	85	2.8	19	5.1	34	4.8	32
			ANAEROBIC					
gamma	516.	89	-		270.	52	190.	37
	51.6	86	-		26.9	52	17.8	34
	5.16	83	-		0.6	11	3.7	72
alpha	375.	89	-		227.	60	109.	29
	37.5	85	-		-	-	31.8	85

used. With the *alpha* enzyme, as in the columns without substrate, only two peaks, the tetrameric and dimeric peaks, were observed. The *gamma* enzyme produced essentially the same pattern under these conditions as was noted with the substrate concentration at 5×10^{-3} M 4-*tert*-butylcatechol (see Table VIII).

4. Chromatography in the Presence of Monophenol.

For consistency, 4-*tert*-butylphenol was chosen to be the monophenolic substrate for the column experiments with monophenol. This substrate was used with 0.02 M phosphate buffer in both the anaerobic and aerobic columns. The results of this work are summarized in Table IX. The only point of difference between the results with this substrate and those obtained with the catecholase substrate is the absence of a monomeric peak in the aerobic columns.

Table VIII:

This table summarizes the results of the aerobic columns which were equilibrated with 5×10^{-4} M 4-*tert*-butylcatechol in 0.02 M phosphate, pH 6.7.

TABLE VIII

Isozyme	Concentration Applied (µg)	Recovery (%)	Tetramer Amount	Tetramer %	Dimer Amount	Dimer %	Monomer Amount	Monomer %
gamma	172.	88	50.9	30	69.8	41	30.6	17
	9.5	83	1.7	18	4.6	48	1.6	17
alpha	375.	91	252.5	67	78.4	21	13.7	3
	37.5	84	18.7	49	13.2	35	-	

Table IX:

This table summarizes the results obtained from the columns, both anaerobic and aerobic, which were equilibrated with 5×10^{-3} M 4-*tert*-butylphenol in 0.02 M phosphate buffer, pH 6.7.

TABLE IX

Isozyme	Concentration Applied (µg)	Recovery (%)	Tetramer Amount	Tetramer %	Dimer Amount	Dimer %	Monomer Amount	Monomer %
			AEROBIC					
gamma	510.	91	352.	69	112.	22	-	
	17.2	85	9.9	57	4.8	28	-	
alpha	375.	88	241.	64	93.	24	-	
	37.5	85	22.	58	10.2	27	-	
			ANAEROBIC					
gamma	510.	89	-		276.	54	175.9	35
	17.2	85	-		4.5	26	10.1	59
alpha	375.	88	-		197.8	53	132.	35
	37.5	85	-		5.1	14	26.7	71

DISCUSSION

A. TYROSINASE

1. Normal Aggregation Equilibrium.

It is well known that tyrosinase exists in solution in at least three aggregation states: monomer, dimer, and tetramer. Of these, the tetramer predominates when no substrate is present (42, 43, 44). The states are interconvertible; the conversion from one form to another is slow. Increasing concentration of the enzyme solution results in a greater percentage of the larger aggregation states (43, 44).

2. Aggregation in the Presence of Substrate.

We have been able to draw several conclusions from our experiments. The addition of substrate favors a shift in the equilibrium toward the less aggregated species (Table X). If the added substrate is a monophenol, the lowest aggregation state found is the dimer. With diphenol, the monomeric subunit is present. The greater the concentration of substrate, the greater the amount of dissociation, but the tetramer is not completely eliminated from the association-dissociation mixture. Each of the species in the polymerization equilibrium is active.

3. Evidence Supporting This Aggregation Equilibrium.

We will now discuss our bases for these proposals, and outline the factors which affect the association-dissociation phenomenon observed with tyrosinase.

a. In the absence of substrate:

We suggest that there exists an aggregation equilibrium for

Table X:

A summary of the results obtained by band centrifugation and with the most dilute enzyme used in the gel filtration experiments compiled to illustrate the influence of substrate on the aggregation equilibrium noted in tyrosinase.

TABLE X

Isozyme	Type of Analysis	Concentration (μg)	Percentage		
			Tetramer	Dimer	Monomer
beta	Band Centrifugation				
	a. no substrate	10-15	no band noted		
	b. with diphenol	10-15	-	-	all
	c. with monophenol	10-15	-	-	all
	Gel Filtration				
	a. no substrate	6.9	92	-	-
	b. with diphenol	17.0	22	52	12
	c. with monophenol	17.2	57	27	-
alpha	Band Centrifugation	10-15			all
	Gel Filtration				
	a. no substrate	37.5	90	-	-
	b. with diphenol	30.0	22	29	35
	c. with monophenol	37.5	58	27	-

tyrosinase and that the aggregated form, the tetramer, is the major species in the absence of substrate. Reviewing the filtration results, in which columns were developed in the absence of substrate (Table II), the dilute enzyme upon elution produced a peak corresponding to the tetramer. The other aggregation states are present, however, as indicated by the columns developed with an increased concentration of enzyme, and from gel electrophoresis (42, 43, 44). It should be noted that, although the tetramer is an active form of tyrosinase, as will be discussed below, it is possible that it is not a form which occurs "*in vivo*". That the aggregation equilibrium observed in the purified enzyme is not an artifact of the preparation procedure has been demonstrated in freshly pressed mushroom juice by gel electrophoresis (42, 44). The tetrameric form, however, may result from the increased concentration of oxygen in the air environment as compared to the natural, intracellular environment. No tetramer was observed in the anaerobic columns. It is suggested, therefore, that some interaction of oxygen with the dimer is essential for formation of the tetramer. Acrylamide gel electrophoresis of the copper depleted enzyme and then readdition of copper, followed by staining with a substrate solution, gives a pattern identical to that obtained with the untreated enzyme (44). Copper is not essential, therefore, to the quarternary structure (44), so that the possibility that oxygen acts as a bridge between the copper ions of dimers to make the tetramer can be ruled out. No other components have thus far been implicated in the protein moiety as being essential to the quarternary structure of tyrosinase, and other groups which might interact with oxygen have not been identified. It appears, at any rate, that the

association of dimer to tetramer is dependent upon the oxygen concentration in the enzymatic environment.

b. In the presence of substrate:

As reported in Results, our sedimentation coefficient values calculated from band centrifugation are 2.51 S in the presence of monophenol and 2.30 S with diphenol. Values reported by other investigators for the slowest sedimenting species of tyrosinase in sodium dodecylsulfate (SDS) have been 2.57, 2.49, and 2.33 S (12). Correcting these values to the reference value, $s_{20,w}$, gave sedimentation coefficients of 2.71 S (12) and 2.70 S (51). Another reported $s_{20,w}$ for the same species produced by 8 M urea is 2.2 S (95). Comparing these values of other workers with our results suggests that the lowest aggregation state of tyrosinase, the monomer, is active under the conditions employed in the ultracentrifugation experiments. The elution pattern from a Sephadex column equilibrated with diphenol produced a peak at molecular weight 30,000 which corresponds to the monomeric molecular weight (12, 45, 51, 81, 86). No significant difference in sedimentation coefficients between monophenolic and diphenolic enzyme complexes is obtained in the centrifugation experiments. However, with aerobic Sephadex columns and monophenol, no monomeric subunit is observed.

Reports in the literature concerning the activity of the monomeric form of the enzyme are in variance. Bouchilloux *et al.* with an SDS dissociated enzyme reported an active monomer (12). Using 8 M urea to obtain the lowest molecular weight species, Zito and Kerterz say their monomer is inactive (95). Robb reports a decreased activity with diphenol and no cresolase activity in his monomer which was obtained by

diethylaminoethyl (DEAE)-Sephadex chromatography (44, 81). Arnaud using band centrifugation and an unpurified enzyme observed only the tetramer (5). Observations made in our laboratory with concentrated enzyme solutions show a relatively inactive protein. Dilution of this enzyme and allowing the dilute solution to stand for an hour increases the observed activity. This suggests a concentration-dependent association to less active forms (43, 44). The identity of the final species is not known, as an octamer and even a dodecamer have been observed with acrylamide-gel electrophoresis in very concentrated enzyme solutions (above 10 mg/ml) (44). With radioactive carbon monoxide, only one copper atom was necessary to complex the diphenol and CO, while with monophenol, one CO molecule complexes with two copper atoms (37). All the evidence presented above supports our conclusion that the monomeric subunit is capable of the dehydrogenation of a diphenol and production of an o-quinone. With monophenols, however, only the band centrifugation work suggested an active monomer, and we conclude, on the basis of the other evidence presented, that the dimer is the smallest active species to react with monophenol. The standard deviation, 0.73, observed in the averages of the sedimentation coefficients obtained with monophenol, is larger than that deviation calculated in the values obtained using diphenol (21). This suggests a possible oscillation between monomer and dimer as the enzyme catalyzes the steps of hydroxylation and dehydrogenation. In this way, the calculated standard deviation of the sedimentation coefficient is made to appear larger than that predicted by theory (21).

We have thus far identified only the smallest active species, and the activity of the other aggregation states including the tetramer

must be evaluated. The Sephadex columns present the best evidence supporting an active tetramer, which evidence is the definition of an elution peak which corresponds to the position predicted for molecular weight 120,000, and also the observation of an o-quinone band which courses through the column in conjunction with the 120,000 molecular weight peak (12, 45, 51, 81, 86). With concentrated *gamma* enzyme and a saturating amount of diphenolic substrate, the sedimentation constant of the observed peak was equal to that of the same enzyme sedimenting without substrate. This peak was symmetrical and distinct in the presence of substrate, and no evidence of other, slower sedimenting molecules was discovered. When a spectrum of this solution was taken after centrifugation, a typical o-quinone spectrum (Figure 3, curve B, Materials and Methods) was obtained proving that the reaction of enzyme with substrate had taken place. Contradictory evidence suggesting that no species of greater aggregation than the monomer is active is provided by the band centrifugation experiments, in which, according to theory, only the heaviest active species will be observed (20, 21, 89, 90, 91). However, in the ultracentrifuge in very dilute enzyme concentrations, alteration in pressure resulting from the high angular velocity of the rotor may affect the association equilibrium in such a way that the predominant species is the monomer. High ionic strengths likewise cause dissociation of tyrosinase, so that the presence of 0.1 M NaCl in the experimental mixture will also influence the equilibrium to favor the monomer (42, 44). Also possible is that the monomer may have the greatest activity of all three aggregation states. The other species may be present in such small concentrations or with such reduced activity in

comparison to the monomer so as not to be noticed by the schlieren optical system (90). Weighing all the evidence, we conclude that, in the association equilibrium, all the aggregation states observed in the presence of a substrate possess enzymatic activity.

c. Mechanism of aggregation:

All forms are eluted from aerobic Sephadex columns equilibrated with diphenolic substrate. Holding the enzyme concentration constant while varying the substrate concentration over a range including 1,000 times its most dilute molarity (5×10^{-4} M) did not change the number or position of peaks (Figure 14). A constant substrate concentration with a steadily decreasing enzyme concentration produces a predominance of monomer. The most dilute enzyme used in conjunction with the most concentrated substrate shows very little tetramer, with the majority of the protein in either the dimeric or monomeric states. More concentrated enzyme (Table VII) in a saturating concentration of substrate is recorded as a diffuse peak which includes the tetramer and dimer. The presence of monomer cannot be proven conclusively in this peak without eliminating the possibility of peak tailing (85). However, the amount of protein in the dimeric position is more than that seen with the same concentration of enzyme in a column without substrate (Table XI). If the presence of substrate alone controlled the dissociation of tyrosinase, the amount of dissociation would be constant, with no further dissociation seen upon application of more dilute enzyme to a column (Table VII). We conclude, then, that the amount of dissociation is directly related to the ratio between substrate and enzyme concentrations, as well as to the type of substrate present, and not to

Figure 14:

These graphs illustrate the results obtained from columns equilibrated with (A) 5×10^{-2} M 4-*tert*-butylcatechol, (B) 5×10^{-3} M 4-*tert*-butylcatechol, and (C) 5×10^{-4} M 4-*tert*-butylcatechol. In graphs A and B, 3.4 μg of β isozyme was applied to each column. In graph C, 9.1 μg of γ isozyme was applied to the column.

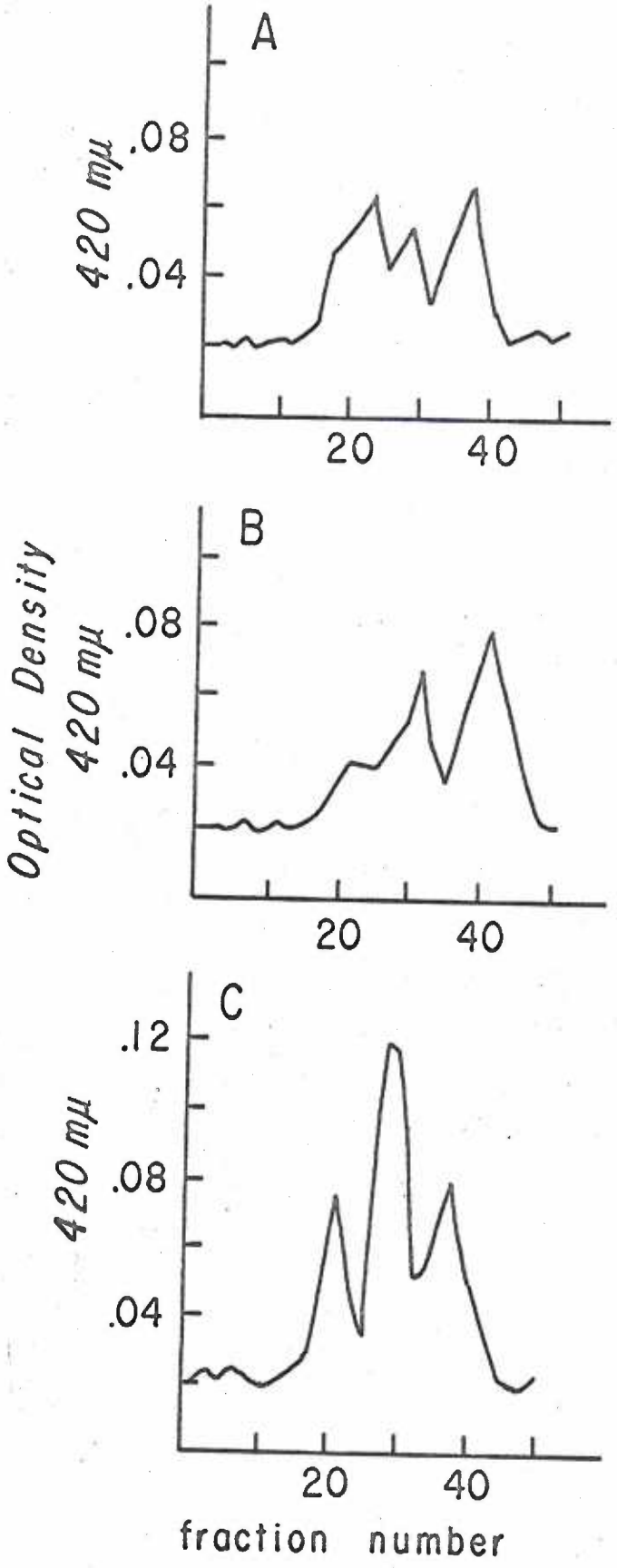
A
.08
.04
420 mμ
20
40
B
Optical Density
420 mμ
.08
.04
20
40
C
.12
.08
.04
420 mμ
20
40
fraction number

Table XI:

An illustration of the various applied concentrations of *beta* enzyme and the results obtained with column equilibrated with substrate compared with control columns without substrate.

TABLE XI

Isozyme	Conditions	Concentration Applied (μg)	Percentage of Tetramer	Dimer	Monomer
beta	No substrate	500.	51	34	8
	With substrate*	500.	34	57	-
	No substrate	171.	34	32	23
	With substrate	172.	18	57	13
	No substrate	6.9	92		
	With substrate	3.4	19	30	36

*5 x 10^{-3} M 4-*tert*-butylcatechol.

the presence of substrate alone. Recall that the elution pattern from columns equilibrated with monophenol showed no monomeric peak.

The association-dissociation equilibrium which exists free of substrate is not concentration dependent in the range, 0-500 µg, which we studied. This means that, as the enzyme concentration decreases, the amount of dissociation does not increase; in fact, the opposite was observed. Our assay technique may not be sufficiently sensitive to record the lower aggregation states, so that they may, indeed, be present in minute quantities at very dilute enzyme concentrations. A concentration-dependent association has been noted in a higher concentration range, above 1 mg/ml (42, 43, 44), affected by ionic strength and heat. No effect on the degree of dissociation was observed when 0.1 M NaCl was added to our equilibration buffers in the column experiments. The graphs relating changes in sedimentation values to the enzyme concentration all have negative slopes, indicative of absent dilution dissociation of the enzyme in the range 1-10 mg/ml. However, this concentration-dependent aggregation is also time dependent (42, 44), so that the negative slopes of the sedimentation velocity graphs are not entirely reliable, and so dissociation or aggregation to states other than the tetramer may exist in these ranges. We would conclude that the association-dissociation observed in our columns without substrate was independent of the enzyme concentration and other factors which affect this equilibrium in the higher concentration ranges.

In the presence of substrate, the equilibrium shifts to favor dissociation into smaller units. The larger the ratio between the substrate and enzyme concentration, the greater the percentage of lower

aggregation states. In a system of extremely dilute protein with a ratio of greater than 5 x 10^{-2} M diphenolic substrate to 3.4 µg of applied enzyme, all the protein might exist as monomer.

Even with our highest ratios (noted above) some of the protein was identified as tetramer. The appearance of tetramer under these conditions may be due to a slow rate of interconversion between the aggregation states even in the presence of substrate. That this is a time dependent process is further proven by the separation of three distinct states during passage through Sephadex G-100, and also by the consistency of the location of these peaks. A system which experiences rapid equilibrium between states exhibits a variable peak position when subjected to Sephadex chromatography (49). No pure aggregation states can be obtained by this method nor by any methods except DEAE-Sephadex chromatography (43, 44, 45, 81), because immediately as enzyme is eluted from the column re-equilibration between the states begins. The rapidity of this association equilibration, analogous to that of *delta*-aminolevulinic acid dehydratase, explains why, despite the substrate-induced dissociation of tyrosinase, no effect has been noted on the kinetic curve (68, 69). According to Frieden, production of an observable allosteric effect requires that the association-dissociation be rapid in comparison with the overall rate of the enzymatic reaction (30). Difference in binding between the monomer, dimer or tetramer could not be calculated from our method. However, since no allosteric kinetics are observed with tyrosinase, the theory of Nichol *et al.* predicts that the binding sites on proteins of all aggregation levels must be equivalent (73). It may be hypothesized that dissociation into active

subunits would result in faster, more efficient oxidation than could be accomplished by the aggregated form. If this is so, the absence of alteration of the kinetic curve is apparently secondary to slow achievement of equilibrium, rather than to a lack of difference in substrate binding between the species (30, 73). In defense of this stand, band centrifugation suggests that the monomer is the most active species. Gel electrophoresis, in the presence of inhibitors such as nitrocatechol, shows an increased proportion of tetramer when compared to a control without inhibitor (42). It appears, therefore, that dissociation into subunits results in more efficient oxidation of substrate by the enzyme.

Band I, the lowest aggregation species observed by gel electrophoresis, has two forms, called I and Ia (42, 43, 44, 81). The sedimentation coefficient of Band I, 3.8 $S_{20,w}$, is larger than those reported earlier for monomeric sedimentation values (12, 44, 51, 81, 95). Injection of this value and the diffusion coefficient of this same species into equation 2 (Materials and Methods) gives a molecular weight of 30,000 for the DEAE-Sephadex produced Band I (44, 81). However, if Band I is assumed to be monomer, the bands observed with gel electrophoresis do not occur as integral values in a log-log plot of mobility vs molecular size (44). Therefore, the absolute identity of Band I is still subject to question (44, 81).

Band I and Ia differ in charge rather than molecular weight (44). Apparently they are inter-convertible (44) and become a single band before associating to higher aggregates, since no inter-convertible tetramers such as Band III and IIIa have been observed with gel electrophoresis (44). If we assume that tyrosinase exists in two monomeric

forms analogous to glutamic dehydrogenase in its dissociated form (29), one of which associates and the other does not, a control mechanism may be advanced for tyrosinase which also explains the observed reaction inactivation obtained with catechol (34, 69, 70).

The presence of diphenolic substrate in the enzymatic environment influences the polymerization equilibrium to favor smaller subspecies of the tetramer--the dimer and the monomer. The monomer exists in two states, one of which, the R state in the terminology of Monod *et al.* (64, 65), is active and is the predominant state when substrate is present. This explains the apparent increased activity of the monomer. After reaction with substrate, however, this R state is converted into the T state (64, 65) which cannot reassociate to form the dimer. The reaction is controlled by the interval of time necessary to convert this T state back to its R form, and then to re-aggregate to the dimer. This time period is, in turn, influenced by the presence of substrate in the immediate enzymatic environment. Intracellularly, no substrate may be in the immediate vicinity of the enzyme, so that it may re-aggregate to dimer at a slow rate. *In vitro*, however, the saturating substrate concentrations used in assay experiments insure a continual supply of R state monomer, both from the R to T transformation and from the association-dissociation equilibrium.

The above mechanism may offer an explanation for the reaction inactivation observed in the presence of catechol (34, 69, 70). In the reaction with catechol, the monomer-catechol-oxygen complex alters the structure of the monomeric unit in such a way to cause it to be changed irreversibly to a T state incapable of reacting with any more substrate

(61, 64), and incapable also of conversion to the R state. The removal of active monomer, R state monomer, from the dissociation mixture causes the reaction to produce more monomer which again is inactivated by its reaction with catechol. Thus a time dependent reaction-inactivation is observed. The length of time needed for complete inactivation to occur is related to the speed of dissociation of tyrosinase into its monomeric subunits and conversion to the T state monomer by reaction with catechol.

4. Summary.

In summary, we observed a non-concentration dependent dissociation of enzyme in the absence of substrate, controlled in part by oxygen, and a concentration-dependent dissociation in the presence of substrate, controlled by the type and concentration of substrate. Initially, an oxygen molecule combines with the enzyme and then substrate is added to the enzyme-oxygen complex (13, 59). This is supported by evidence from anaerobic Sephadex chromatography, which showed no difference in the elution patterns of products from the substrate free column upon addition of either monophenolic or diphenolic substrate to the equilibrium buffer. The oxygenated enzyme ($Cu\text{-}O_2$), either tetramer, dimer, or monomer, then complexes with a diphenol to produce the o-quinone (13). After catalysis of the dehydrogenation reaction, the enzyme, still joined with the remaining oxygen atom (Cu-O), then combines with both diphenol and monophenol to catalyze the simultaneous production of diphenol and o-quinone (13, 61). This reaction is mediated by either the dimer or the tetramer. The necessity of existence of a higher aggregation state, and the time dependency of this aggregation, could partly explain the lower specific activity with respect to monophenolic

substrate of each of the isozymes, *beta*, *gamma* and *delta*, when compared to their oxidation of diphenol.

B. ALPHA AS A DISTINCT PROTEIN

1. Sedimentation Evidence.

That the *alpha* isozyme is a completely separate protein has already been proposed (45). The results reported here appear to support this proposition. The S_{20} at infinite dilution observed for *alpha*, 6.62 S, is distinctly lower than those obtained for the other isozymes. The *beta*, 7.15 S, and *gamma*, 7.10 S, isozymes have nearly equal S_{20} values. Values for both *beta* and *gamma* of 7.25 $S_{20,w}$ at infinite dilution reported by Bouchilloux *et al.* agree with the results reported here (12, 45). The *delta* isozyme, 7.29 S, has a higher S_{20} at infinite dilution, but it was extrapolated from only two experimentally produced values of the sedimentation coefficient, and more are necessary for a more definitive identification of this constant. It appears, however, that the *delta* value will be very close to those reported for the *beta* and *gamma* isozymes (12, 45).

2. Chromatography Behavior.

There are reported differences in the amino acid composition of *alpha*, as compared to *beta*, *gamma* and *delta* (44). The *alpha* isozyme also acts in a way unique from the other isozymes during Sephadex chromatography. With no substrate, in air, the *alpha* isozyme, even in the highest concentrations, does not show a monomeric peak. In the anaerobic column, however, the pattern is the same as that obtained

from the other isozymes, with the exception that, with the most dilute *alpha* enzyme in anaerobic diphenolic buffer, no dimeric peak is seen. Possibly as a result of the very small amount of *alpha* present in these columns, of the reduced catecholase activity of *alpha*, and of the greater sensitivity of *alpha* to denaturing procedures (42, 43, 44), the dimer, although quite probably present, was not seen. The absence of all but a small monomeric peak when concentrated *alpha* was applied to the aerobic columns equilibrated with dilute diphenol (Table IV) again strongly suggests a distinct difference between *alpha* and the other isozymes. Conclusive evidence might be had following determination of diffusion coefficients for each of the four isozymes, in order to obtain their absolute molecular weights, and this has yet to be done. However, on the basis of evidence cited before, together with that presented in this work, we conclude that *alpha* is a separate protein from the *beta*, *gamma* and *delta* isozymes, even though it mediates the same reactions.

SUMMARY

This work demonstrates, by two different methods, an enzymically active monomer of tyrosinase, at least in the presence of diphenol. Since Kubowitz's work in 1938 with carbon monoxide-copper ratios (55), most theories have assumed two atoms of copper at each active site of the tyrosinase enzyme. The most recent carbon monoxide binding work using radioactive CO suggested that this was not the case when diphenol was used as a substrate (37). Our work, reported here, has confirmed the recent CO conclusions: that the hydroxylation step, described as the cresolase activity of tyrosinase, is catalyzed by the tetrameric or dimeric form of the enzyme, while the dehydrogenation step, the catecholase activity, can be catalyzed by the tyrosinase monomer. Our work, described in this paper, elucidates these mechanisms. We also confirm that both reactions are catalyzed by the same enzyme (59, 62, 95).

There are two different means of activating oxygen for enzymatic action. One reaction is that to produce the diphenol, in which two copper ions accomodate each oxygen molecule; the second reaction results in the o-quinone, requiring only one copper per oxygen at the active site.

The *alpha* isozyme has been shown by us to be distinctly different in molecular weight and in elution pattern during chromatography on Sephadex G-100 from the other isozymes. This confirms the earlier work concerning its amino acid composition (45), and points to the fact that the *alpha* "isozyme" is a separate protein, and not an isoenzyme of mushroom tyrosinase.

A possible reaction mechanism is proposed which accounts for the interaction of all the polymeric forms of tyrosinase, and may explain the reaction inactivation observed in the tyrosinase-oxygen-catechol reaction.

Finally, a method of reaction analysis is presented which offers an ideal system for the study of the polymerization phenomenon found in tyrosinase, and for the determination of rate constants and of factors controlling this dissociation equilibrium.

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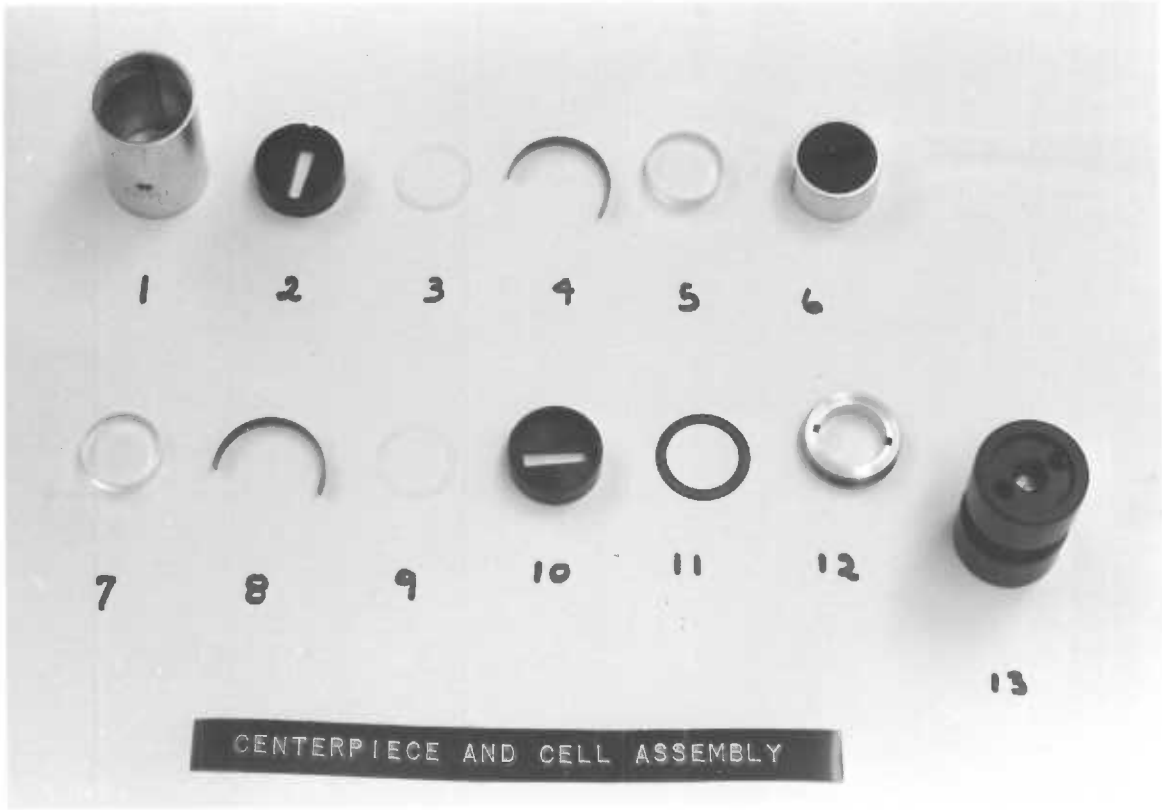
APPENDIX

EXPERIMENTAL PROCEDURE FOR BAND CENTRIFUGATION

10-15 µl of enzyme solution were added to the sample cavity with 0.01 or 0.05 µl Hamilton syringe (89). Care was taken to avoid the formation of air pockets in the sample hole. The cell components were then assembled (Figure 15) and secured with a Beckman torsion wrench (8) to 135 pounds. Next 0.55 ml of substrate solution were added to the center-well and the gasket and plug tightened. It was necessary that the counter balance differ by no more than 0.5 grams from the assembled cell so that, if the centerpiece were to leak during centrifugation, the rotor would not become dangerously unbalanced and disengage from the rotor drive shaft. The cell and counterbalance were then aligned in the rotor. After attachment of the rotor to the drive shaft, the chamber was closed and the vacuum pump was started, followed after several minutes by the activation of the diffusion pump. Before initiation of the rotor drive, a mercury vapor light was provided. Cooling of the diffusion pump and the light source was accomplished by a tap water bath. An AnD rotor with a maximum speed of 59,780 r.p.m.'s was used exclusively in this procedure (8). With the Schlieren analyser angle set at 50°, layering of the enzyme was observed immediately after starting the rotor. This initial peak was small, due to the difference between the densities of the enzyme solution and of the solvent. As this gradient is soon equalized, the initial peak is lost. After the desired speed, 59,780 r.p.m.'s, was reached, photographs were taken at 16 minute intervals until, after six exposures, the rotor drive was shut

Figure 15:

This picture illustrates the various components of an ultracentrifuge cell. They are as follows: 1. the cell housing; 2. bottom window holder; 3. polyethylene gasket fitting between the window and the window holder; 4. a bakelite gasket which fits around the window and holds it in place; 5. the cell windows, either quartz or sapphire; 6. the centerpiece as illustrated in Figure 3; 7, 8, 9, and 10. the components which comprise the top window holder and window; 11. the gasket fitting between the top window holder and the housing screw; 12. the screw fitting into the cell housing enabling it to be aligned and kept in position during the centrifugation; and 13. the counterbalance. These pieces are numbered in the order which they are assembled into the complete cell for centrifugation.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CENTERPIECE AND CELL ASSEMBLY

off and braking was begun. The light source and the diffusion pump were discontinued as soon as exposure of the final plate had been accomplished. At least 5 minutes must have elapsed after the shutdown of the diffusion pump before opening of the chamber. When the rotor came to rest, the vacuum pump was disengaged and the vacuum in the chamber released. Three minutes must pass, to insure complete release of the vacuum, before the hoist could be activated to open the chamber. The rotor having been removed from the chamber, the vacuum pump was again called upon to remove condensed moisture from the chamber.

The rotor must be wiped clean and each of the cell components carefully cleaned and wrapped in preparation for storage after each experimental run.