PART I

STUDIES ON THE ACTIVATION OF *DROSOPHILA* PHENOL OXIDASE

PART II

STUDIES ON SERUM INSULIN IN NORMAL SUBJECTS AND DIABETICS

Thesis by

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This thesis is

dedicated to my

parents.

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ABSTRACT

PART I

Phenol oxidase is the enzyme responsible for hardening and pigmentation of the insect cuticle. In Drosophila, phenol oxidase is a latent enzyme. Enzyme activity is produced by the interaction of a number of protein components. A minimal activation scheme consisting of six protein components, designated Pre S, S activator, S, P, P' and A_1, is described. Quantitative assays have been developed for the S activator, S, P and P' proteins and these components have been partially purified. Experiments describing the interactions of the six components have been conducted and a model for the activation of phenol oxidase in a minimal system is proposed. Possible mechanisms of the reactions between the constituents of the activating system and potential regulatory mechanisms involved in phenol oxidase production and function are discussed.

ABSTRACT

Part II

A method has been developed for the partial purification of insulin from human serum. A procedure for the determination of the electrophoretic mobility of serum insulin on polyacrylamide gels is described. An electrophoretic analysis of insulin isolated from a normal subject is reported and in addition to a major band, the existence of a number of minor bands of immunoreactive insulin is described. A comparison of the electrophoretic patterns of insulin isolated from normal and diabetic subjects was carried out and indications that differences between them may occur are reported.

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STUDIES ON THE ACTIVATION OF *DROSOPHILA* PHENOL OXIDASE

INTRODUCTION

Section 1. General.

Drosophila has long been recognized as a valuable tool in the study of genetics in eukaryotic organisms. Although the possibilities of *Drosophila* as a system for the study of development and differentiation were realized long ago by Stern, Hadorn and others (53), interest in the developmental biology of *Drosophila* has increased in recent years.

Phenol oxidase is a complex, developmentally controlled enzyme in *Drosophila* (30, 33, 34, 35, 40) and is a potentially useful tool for the study of problems concerned with the developmental control of enzyme activity and with the control of cuticular biosynthesis. This thesis is concerned with a biochemical analysis of phenol oxidase as a prelude to studies of its developmental regulation.

The comparative biochemistry of phenol oxidase has been extensively reviewed on several occasions (1, 2, 38, 54).

Section 2. Reactions and Nomenclature.

Phenol oxidase (o-diphenol: O_2 oxidoreductase, E.C.1.10.3.1) catalyzes the ortho hydroxylation of monophenols and the production of ortho quinones from ortho diphenols. The enzyme has been called tyrosinase, phenolase, phenolase complex, dopa oxidase, polyphenol oxidase, cresolase, and catecholase (2).

Section 3. Function.

A. Insects.

Phenol oxidase is involved in pigmentation and hardening of the insect cuticle (36). This function is mediated through the production of quinones derived from the oxidation of phenolic compounds (37). The resultant highly reactive quinones can enter into polymerization reactions and also cross link polypeptide chains of cuticular proteins (38). These reactions result in hardening and pigmentation of the integument (39). In addition to its enzymatic function in cuticular synthesis it has been proposed that phenol oxidase may also play a structural role in the formation of cuticle (40).

Phenol oxidase functions unrelated to cuticular synthesis have also been proposed. These include wound healing (46) and defense against invading parasites (47).

B. Plants.

The phenol oxidase function which has received the most attention in plants is the browning reaction following injury. The suggestion has been made that this reaction is connected with wound healing and antibiosis (51). The enzyme has also been implicated in the biosynthesis of lignins, tannins, alkaloids and flavonoids and in the hardening and browning of seed coats, spores and barks (38).

C. Vertebrates.

Phenol oxidase is the enzyme responsible for melanization in vertebrates (52). Melanins in vertebrates are found in specialized cells called melanocytes, in the form of granules with a well defined structure (41).

Section 4. Latency.

A. General.

Phenol oxidase is observed to occur in a latent form in many species. This may be partly due to problems of transport. Phenol oxidases are often observed to occur in a highly aggregated insoluble state which may be necessary for function. Transport of the enzyme to its site of action would be facilitated by deposition of soluble, lower molecular weight precursors followed by activation and aggregation in situ. Protection against the extremely reactive and often toxic quinones produced by phenol oxidase may also account for the maintenance of the enzyme in an inactive form when not required.

B. Vertebrates.

Only a few examples of latent phenol oxidases have been described in vertebrate systems. This may be partly due to difficulties involved in extracting phenol oxidases from the tightly organized melanin granules where phenol oxidase activity is located.

A soluble precursor of phenol oxidase can be extracted

from the skin of the newt, Triturus cristatus. This enzyme can be activated by anionic detergents (3).

There is a report on the activation of phenol oxidase in extracts of Rana pipiens eggs (4). A variety of denaturing agents including chloroform vapor, urea, anionic detergents, and urethane result in the appearance of enzyme activity. A soluble phenol oxidase precursor has been extracted from the skin of adult Rana pipiens. The enzyme is activated by treatment with trypsin (5).

There are few data bearing directly on the question of activation of latent phenol oxidases in the mouse. Work by Moyer (41), however, has suggested that elaboration of phenol oxidase activity in the melanin granules of mouse melanocytes may be the result of a complex aggregation of proteins. He studied the development of melanin granules and observed that granule development consists of the aggregation of subunits into fibers and the cross linking of these fibers to form a sheet-like matrix which contains phenol oxidase activity. The formation of the matrix is seen to coincide with the deposition of melanin at periodic intervals along the matrix. Alleles at three loci, B, C and P are seen to alter the sequence of development in a manner suggesting that they are the structural genes for different macromolecules contained in the subunits. The suggestion is made that the subunit of the granule is a complex aggregate of molecules containing phenol oxidase activity.

C. Fungi.

Although considerable research has been done on fungal phenol oxidases there are few data concerning activation reactions.

No phenol oxidase precursor has been isolated from Neurospora, however there is an observation of an increase in phenol oxidase activity, exhibiting first order kinetics, in extracts of Neurospora (6).

In fresh extracts of the ascomycete, Podospora anserina phenol oxidase exists in a latent form (7). Activation is observed on purification or heating. The latent and active forms of the enzyme have different electrophoretic mobilities but the same sedimentation constant.

D. Insects.

1. General.

Phenol oxidase has been observed to occur in a latent form in all systems studied. In those systems in which the process has been well characterized, the activation seems to be quite similar.

2. Melanoplus.

A number of papers have been published by Bodine and co-workers describing the activation of phenol oxidase in eggs of the grasshopper Melanoplus differentialis (8, 9, 10, 11, 12, 13). No phenol oxidase activity is observed in fresh extracts of Melanoplus eggs. Phenol

oxidase activity appears in homogenates following a period of incubation at 0°C. If the extract is centrifuged prior to the appearance of enzyme however, the process of activation is blocked and the proenzyme is obtained in a soluble, stable form. This proenzyme can be activated by mixing it with the lipid layer removed by centrifugation or by a variety of denaturing agents including heat, urea, anionic detergents, organic solvents, shaking and heavy metals. On the basis of these results Bodine suggested that the *in vivo* activation process involves a partial denaturation of the proenzyme. It is difficult, however to rule out the possibility that a proteinaceous activating factor is involved. In most other insect systems which have been studied the presence of a protein activating factor seems to be indicated. Evidence suggests that in several systems it is itself latent. It would seem possible that the failure to demonstrate a protein activating factor in this system can be attributed either to a failure to extract part of an apparently complex activating system or to the denaturation of some component of it. The activation by denaturing agents may be an artefactual phenomenon.

3. *Tenebrio*.

Phenol oxidase in extracts of *Tenebrio molitor* last instar larvae also exists as a soluble proenzyme (14). The proenzyme can be maintained in a latent

form by centrifugation or chromatography on Sephadex G-25. An activating factor which is stable to heating at 100°C. is found in the centrifugal precipitate. The proenzyme was activated by 16 to 22 carbon fatty acids isolated from the larval integument. No evidence was found for a protein activator but as in the case of the Melanoplus enzyme, negative evidence cannot be regarded as conclusive.

4. Musca.

A soluble proenzyme can be isolated from Musca vicina at all ages (15). Activation occurs with sigmoidal kinetics following a concentration dependent lag period. Proenzyme has been prepared in a stable form from prepupae, which lack activating capacity. Activator free of proenzyme has been prepared by ammonium sulfate fractionation of pupal extracts (16). In the course of kinetic experiments Ohnishi has shown that an increase in activator concentration occurs during phenol oxidase activation in crude extracts (17). Ohnishi suggested that this fact supports an autocatalytic mechanism but the results can also be explained by assuming that the activator occurs in a latent form which undergoes an activation reaction. A Lineweaver-Burke plot of the kinetics of activation with proenzyme and activator suggest a simple enzyme substrate relationship (18). Sodium picryl sulfate, N-bromosuccinimide and iodide block the activation by irreversible inhibition

of the activator. These compounds have no effect on the proenzyme. The activator is heat labile, non dialyzable and unstable to pH below 6 or above 9. The active enzyme tends to aggregate and can be removed from solution by centrifugation.

5. Calliphora.

Karlson and co-workers have conducted extensive investigations of Calliphora erythrocephala phenol oxidase. Fresh extracts are devoid of phenol oxidase activity. Activity appears in extracts on incubation at $0^{\circ}C$. following a concentration dependent lag period. The activation process exhibits sigmoidal kinetics (19). The kinetics of activation have been analyzed at varying concentrations and were found not to fit well with the theoretical curves for an autocatalytic process. The suggestion was made that the sigmoidal activation kinetics could be accounted for by assuming that the activator exists as a latent precursor. It has been reported that the activator, which is located in the integument and the proenzyme, isolated from the hemolymph have been purified (21). The criteria of purity however are not satisfactory. When purified activator and proenzyme are mixed, activation follows linear kinetics with no lag period. The proenzyme can be activated by α-chymotrypsin and aminopeptidase (20). The suggestion is made that the natural activation process

involves limited proteolysis. A conclusive demonstration of this hypothesis will have to await the demonstration that the natural activator can act in a proteolytic fashion on the proenzyme to produce active phenol oxidase.

An interesting experiment in this system suggests that the synthesis of the activator is controlled by ecdysone, the moulting hormone (22). Destruction of the ring gland results in a sharp decline in activator concentration. Injection of ecdysone results in an increase in the concentration of the activator to its normal level within 2 days. Ecdysone itself does not act on the proenzyme in vitro (23).

Work in Calliphora stygia has shown the activator to be located in the integument in this species as well (24). Activator is found in the cuticular lining of the salivary duct but not in the salivary gland cells themselves. Activator isolated from Drosophila was found to activate the Calliphora proenzyme.

6. Bombyx.

Activation of phenol oxidase from the hemolymph of Bombyx mori also occurs with sigmoidal kinetics following a concentration dependent lag period (25). Activation is accompanied by aggregation of the active enzyme. The activator, located primarily in the integument, and the proenzyme, found in the hemolymph, can be separated by ammonium sulfate fractionation. When activator and proenzyme are mixed, phenol oxidase activity is produced with

no lag period and linear kinetics. The pH optimum of the activation process is around pH 6.0. The reaction is inhibited at high ionic strength. The proenzyme can also be activated using α-chymotrypsin or semi alkaline protease of Aspergillus melleus, however the enzyme produced differs in several ways from the product of the natural activation system. The active enzyme is about half the molecular weight of the proenzyme and no aggregation occurs. The product of the artificial activation also has no monophenol oxidase activity and lacks stereospecificity in reactions with diphenols (26). In addition, less than 10% of the potential activity of the proenzyme is released (26). The suggestion is made that the natural activation involves limited proteolysis, however no evidence has been presented linking the artificial activation with the in vivo process.

Activation proceeds in a similar manner in the Chinese oak silkmoth, Antheraea pernyi (27). A latent enzyme is found in extracts of Antheraea and activation proceeds in a sigmoidal fashion. The activation reaction has two pH optima and the suggestion is made that the activation reaction occurs in at least two steps. An increase in activator concentration with time is observed in the crude extract and the suggestion is made that the activator is formed from a precursor (28). It is also noted that if the rate of the activation reaction varies linearly as a function of activator concentration, then the linear

formation of activator would be sufficient to account for the sigmoidal kinetics observed.

Before proceeding to a discussion of phenol oxidase in Drosophila it is of interest to summarize the problem as observed in the variety of insect systems discussed. In all of the systems it was found that phenol oxidase was a latent activity, being absent in fresh homogenates. In most of the cases discussed it was concluded that the enzyme was formed by the action of a protein activator on a proenzyme. Kinetic analysis of activation suggested a potentially more complex situation and the possibility that the activator itself was latent was discussed. In fact however, the activating system was not resolved in sufficient detail in any of the systems to draw any firm conclusions concerning the activation process. Without such resolution it is impossible to determine the number of components which might be involved in the production of phenol oxidase. Isolation of a fraction which causes the activation of a proenzyme is not sufficient grounds to claim that the fraction contains a single activating component. An understanding of the production of phenol oxidase must depend on a definition of the system at the component level. This would involve fractionation of the components involved prior to the onset of activation. The work of Mitchell, described below, on the fractionation of the components of the Drosophila system will provide a background for the work presented in this thesis.

7. Drosophila.

Phenol oxidase has been known in Drosophila since the work of Graubard (29). Horowitz and Fling, working with adult Drosophila melanogaster showed that phenol oxidase activity is latent in fresh extracts and following a concentration dependent lag period, activity appears with sigmoidal kinetics (30). The reaction kinetics fit the theoretical curve for an autocatalytic process and Horowitz proposed that a molecule of activator reacts with a molecule of proenzyme to produce a molecule of active enzyme and another molecule of activator. As in other systems, the active enzyme aggregates and can be removed from solution by centrifugation.

Ohnishi (31) demonstrated that a similar situation occurs in the larvae of Drosophila virilis. Fresh extracts contain no activity and activation occurs with sigmoidal kinetics.

Lewis and Lewis (32) demonstrated that the presence of lipid affects the in vitro process of activation.

Mitchell has investigated the activating system in detail. Ammonium sulfate fractionation and disc electrophoresis have been utilized to demonstrate the existence of at least five protein components, designated A_1, A_2, A_3, S and P, which participate in the activation reaction.

The A components can be visualized on disc electrophoresis gels which have been preincubated in S and P

followed by incubation in substrate. A_1 produces an enzyme with mono- and diphenol oxidase activity while the A_2 and A_3 components produce an enzyme with only diphenol oxidase activity.

Using dissection techniques, S activity has been localized in the larval salivary gland (34). As noted above, similar work on the larger Calliphora stygia salivary gland has located activator in the cuticular lining of the salivary duct but not in the salivary gland cells themselves. The suggestion has been made that S plays a catalytic role in the formation of phenol oxidase since it influences activation time but not maximal activity. A procedure for the partial purification of S has been published (34). S activity has been shown to be absent in 4 to 8 hour prepupae, accounting for the apparent lack of phenol oxidase activation at this stage (35). It has been demonstrated that S activity is latent (34).

Section 5. Research Outline.

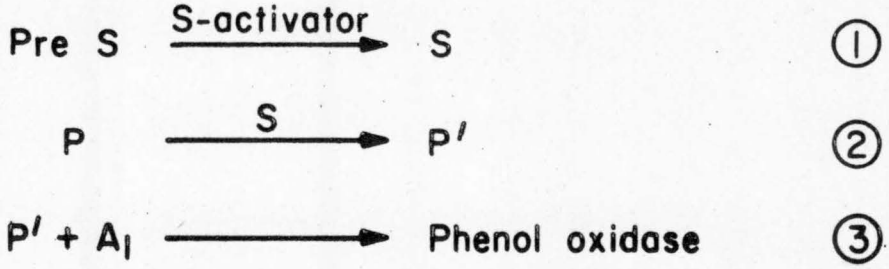
The work of Mitchell et al. has shown that the activation of phenol oxidase in extracts of Drosophila involves the complex interaction of a number of components. The objective of the work presented in this thesis was to define a minimal activation scheme and to determine the nature of the interactions between the various components involved in such a scheme. It was clear at the outset that this would involve purification of the components concerned, at least

to the extent that the various constituents of the system could be shown not to be cross contaminated with each other. The finding of methods for handling and studying the various substances involved depended on the development of quantitative assays specific for each component.

The results presented in this thesis suggest that a complex series of reactions is involved in the activation. A model of phenol oxidase activation consistent with the work of Mitchell et al. and with the work presented here is shown in Figure 1, as a framework for sorting out the components of the system. In this scheme, 6 components are shown interacting in an orderly sequence of reactions to produce active enzyme. The first step in the sequence is the conversion of the precursor of S, Pre S, to S. This reaction is catalyzed by a component designated the S activator. The S formed in this reaction then acts catalytically on the P component to form P' which in turn reacts with A_1 to produce phenol oxidase. A_2 and A_3 are not observed to be necessary in this sequence but it is assumed that, in addition to A_1, these components do participate in the activation process in vivo and in crude extracts.

This thesis is concerned with the purification of the components mentioned in the proposed reaction sequence and with an analysis of the reactions which they undergo.

Figure 1. A model of a minimal system for the activation of phenol oxidase.


Pre S
S-activator
S
①
P
S
P′
②
P′ + A1
Phenol oxidase
③

MATERIALS AND METHODS

1. Drosophila Culture.

Drosophila melanogaster Oregon R eggs were collected and cultured according to the mass culture technique of Mitchell and Mitchell (42). Animals were collected on the sixth day after egg laying when at least 90% had formed puparia. These animals are referred to as 24 hour pupae. The pupae were washed and dried on filter paper and then frozen in liquid nitrogen and stored at -70°C. In some experiments, 4 to 8 hour prepupae were used. These were carefully staged by taking advantage of the fact that an air bubble is secreted 4 hours after puparium formation which causes animals at this age to float. Mixed larvae and prepupae were put in distilled water and any floaters were discarded. The other animals were collected, dried and incubated at 25°C for three hours. They were again placed in distilled water and the floaters were collected and dried on filter paper. The animals thus selected were inspected under a dissecting microscope and asynchronous animals were discarded. The staged animals were stored frozen at -70°C.

2. Phenol Oxidase Assays.

Phenol oxidase was measured as described by Mitchell (35). One unit of phenol oxidase in this assay is defined

as that amount for which the $\Delta OD_{475\ m\mu}$./10 minutes is equal to 1.0.

3. Temperature.

Unless indicated otherwise all procedures were carried out at 0 to 4°C.

4. Gel Filtration.

Bio-Gel P series gels were prepared as recommended by the manufacturer (43). Columns were poured at exactly the same hydrostatic pressure they were to be run at. Fractions were collected volumetrically.

5. Hydroxylapatite Chromatography.

Bio-Gel HT was prepared exactly as recommended by the manufacturer (43). Pasteur pipets were used for one ml. columns. For larger bed volumes small glass columns were used. Glass wool was used for a bed support. Pressure was applied to the columns using a rubber bulb.

6. Preparation of H.*

H is a 36-56% ammonium sulfate fraction used in the assay of the S component. Four grams of 4 to 8 hour prepupae were ground to a smooth paste in a mortar and pestle

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*Based on a procedure suggested by H.K. Mitchell.

with 2 g. of sand and 8 ml. of 0.1 M phosphate buffer pH 6.3. The paste was diluted with 40 ml. of 0.1 M phosphate pH 6.3 and centrifuged at 30,000 r.p.m. for 10 minutes in the Type 40 rotor of the Beckman model L ultracentrifuge. The supernatant was filtered through nylon mesh and brought to 36% saturated $(NH_4)_2SO_4$ by the addition of saturated $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 25,000 r.p.m. for 25 minutes in the Type 30 rotor. The supernatant was brought to 56% saturation and the pellet was collected by centrifugation at 25,000 r.p.m. for 25 minutes in the Type 30 rotor. The pellet was dissolved in 4 ml. of 0.02 M phosphate pH 6.3. The solution was desalted by passage over a 10 ml. Sephadex G-25 column. Four ml. were discarded and 6 ml. were collected. The H was stored frozen in 250 µl. aliquots at -70°C.

7. Preparation of A_1.

Ten grams of 24 hour pupae were ground to a smooth paste in a mortar and pestle with 5 g. of sand and 20 ml. of 44% saturated $(NH_4)_2SO_4$. The paste was diluted with 80 ml. of 44% $(NH_4)_2SO_4$ and centrifuged at 25,000 r.p.m. in the Type 30 rotor for 20 minutes. The supernatant was filtered through nylon mesh and brought to 56% saturation by the addition of a solution of saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 25,000 r.p.m. for 20 minutes in the Type 30 rotor. The pellet was

dissolved in 10 ml. of 0.01 M phosphate pH 6.7 and the solution was brought to 44% saturated $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 25,000 r.p.m. for 20 minutes in the Type 30 rotor. The supernatant was brought to 56% $(NH_4)_2SO_4$ and the precipitate was collected by centrifugation at 25,000 r.p.m. for 20 minutes. The pellet was dissolved in 2 ml. of 0.01 M phosphate pH 6.7 and dialyzed overnight against 500 ml. of the same buffer. The A_1 preparation was stored frozen at -70°C in 250 µl. aliquots.

8. Preparation of Pre S.

One gram of 4 to 8 hour prepupae was ground to a smooth paste in a mortar and pestle with 0.5 g. of sand and 2 ml. of 0.1 M phosphate pH 6.3. The paste was diluted with 8 ml. of 0.1 M phosphate pH 6.3 and centrifuged at 30,000 r.p.m. for 5 minutes in the Type 40 rotor. The supernatant was filtered through nylon mesh and brought to 36% saturated $(NH_4)_2SO_4$ by the addition of a saturated solution of $(NH_4)_2SO_4$. The pellet was collected by centrifugation at 25,000 r.p.m. for 20 minutes in the Type 30 rotor. The pellet was dissolved in 2 ml. of 0.01 M phosphate pH 6.7 and 250 µl. aliquots were stored frozen at -70°C.

9. Preparation of S Activator.

A. Ammonium Sulfate Fractionation.

One gram of 24 hour pupae was ground to a smooth paste in a mortar and pestle with 0.5 g. of sand and 2 ml. of 0.1 M phosphate pH 6.3. The paste was diluted with 8 ml. of 0.1 M phosphate pH 6.3 and centrifuged for 5 minutes at 30,000 r.p.m. in the Type 40 rotor. The supernatant was filtered through nylon mesh and brought to 36% saturated $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 25,000 r.p.m. for 20 minutes in the Type 30 rotor. The supernatant was brought to 56% saturated $(NH_4)_2SO_4$ and the pellet was collected by centrifugation at 25,000 r.p.m. for 20 minutes in the Type 30 rotor. The pellet was dissolved in 1 ml. of 0.01 M phosphate pH 6.7.

B. Hydroxylapatite Chromatography.

S activator (200 µl.), prepared through ammonium sulfate fractionation as described in part A was applied to a hydroxylapatite column with a 1 ml. bed volume equilibrated with 0.01 M phosphate pH 6.7. The column was eluted with a step gradient of increasing concentrations of phosphate buffer. Fractions (2 ml.) were collected and assayed for S activator (see Results, section 1B). The $OD_{280\ m\mu}$. of the fractions was measured.

10. Preparation of S.

A. Ammonium Sulfate Fractionation.

Ten grams of 24 hour pupae were ground to a smooth paste in a mortar and pestle with 5 g. of sand and 20 ml. of 35% saturated $(NH_4)_2SO_4$. The paste was diluted with 105 ml. of 35% $(NH_4)_2SO_4$. The homogenate was centrifuged for 30 minutes at 9,500 r.p.m. in the GSA rotor of the Servall centrifuge. The supernatant and lipid layer were discarded. The pellet was washed by resuspension in 125 ml. of 35% $(NH_4)_2SO_4$ and collected by centrifugation at 9,500 r.p.m. in the GSA rotor for 30 minutes. The washing was repeated once. Following the second wash the pellet was extracted with 45 ml. of 0.01 M phosphate buffer pH 6.7 and centrifuged at 25,000 r.p.m. for 5 minutes in the Type 30 rotor of the Beckman model L ultracentrifuge. The supernatant was immediately brought to 35% saturation by the addition of saturated $(NH_4)_2SO_4$ and centrifuged at 25,000 r.p.m. for 20 minutes in the Type 30 rotor. The pellet was dissolved in 5 ml. of 0.01 M phosphate pH 6.7 and centrifuged at 30,000 r.p.m. for 15 minutes in the Type 40.2 rotor. This supernatant is referred to as crude S.

B. Hydroxylapatite Chromatography.

Crude S (5 ml.) was prepared as described above

and applied to a 5 ml. hydroxylapatite column equilibrated with 0.01 M phosphate pH 6.7. The column was eluted with 10 ml. of 0.1 M phosphate pH 6.3 followed by 8 ml. of 0.02 M phosphate pH 6.7. Fractions (2 ml.) were collected and the $OD_{280\ m\mu}$ was measured. Aliquots (25 µl.) were assayed for S activity and for P' activity (see Results, section 1, parts C and E).

11. Preparation of P.

A. Ammonium Sulfate Fractionation.

Two grams of 4 to 8 hour prepupae were ground to a smooth paste in a mortar and pestle with 1 g. of sand and 4 ml. 0.1 M phosphate pH 6.3. The paste was diluted with 16 ml. of 0.1 M phosphate pH 6.3 and the homogenate was centrifuged at 30,000 r.p.m. for 5 minutes in the Type 40 rotor. The supernatant was filtered through nylon mesh and brought to 35% saturated $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 25,000 r.p.m. for 25 minutes in the Type 30 rotor. The supernatant was brought to 50% saturated $(NH_4)_2SO_4$ and the pellet was collected by centrifugation at 25,000 r.p.m. for 25 minutes. The pellet was then dissolved in 3 ml. of 0.01 M phosphate pH 6.7.

B. Hydroxylapatite Chromatography.

An ammonium sulfate fraction (3 ml.) was prepared

as described in part A and immediately applied to a 5 ml. hydroxylapatite column equilibrated with 0.01 M phosphate pH 6.7. The column was eluted with 10 ml. of 0.05 M phosphate pH 6.7 and 10 ml. of 0.1 M phosphate pH 6.7. Fractions (2 ml.) were collected and 25 µl. aliquots were assayed for P activity (see Results, section 1D). The $OD_{280\ m\mu}$ was measured.

C. Bio-Gel P-60 Chromatography.

An ammonium sulfate fraction (3 ml.) was prepared as described in part A and applied to a 2.5 x 80 cm. P-60 column. The column was eluted with 0.01 M phosphate pH 6.7 at a flow rate of 8.5 ml./hour. Fractions (4.5 ml.) were collected and 50 µl. aliquots were assayed for P activity. The $OD_{280\ m\mu}$ of the fractions was measured.

12. Preparation of P'.

Two methods were used for the preparation of P'. In the first method P' was prepared by the reaction of partially purified S and P and in the second P' was isolated from a crude S preparation.

Method I.

One ml. of P partially purified through ammonium sulfate fractionation and hydroxylapatite chromatography (Materials and Methods, section 11) was incubated with

0.4 ml. of S partially purified through ammonium sulfate fractionation and hydroxylapatite chromatography (Materials and Methods, section 10) for at least 90 minutes.

Method II.

A. Ammonium Sulfate Fractionation.

An ammonium sulfate fraction was prepared from 10 g. of 24 hour pupae exactly as described in section 10A above.

B. Bio-Gel P-60 Chromatography.

An ammonium sulfate fraction (5 ml.), prepared as described in part A above, was applied to a 2.5 x 80 cm. column. The column was eluted with 0.01 M phosphate pH 6.7 at a flow rate of 8.5 ml./hour and 4.5 ml. fractions were collected. Fractions were assayed for P' activity (Results, section 1E) and the $OD_{280\ m\mu}$ was measured. Fractions containing P' activity were pooled and concentrated by ultrafiltration.

C. Bio-Gel P-30 Chromatography.

An ammonium sulfate fraction (2 ml.), prepared as described in part A above, was applied to a 2.5 x 35 cm. P-30 column. Fractions (3 ml.) were collected and assayed for P' activity. The $OD_{280\ m\mu}$ was measured.

D. Hydroxylapatite Chromatography.

The pooled and concentrated fractions (2 ml.)

of the P-60 column (part B) were applied to a hydroxylapatite column with a 1 ml. bed volume, equilibrated with 0.01 M phosphate pH 6.7. The column was eluted with 3 ml. each of 0.02 M phosphate, 0.05 M phosphate and 0.1 M phosphate pH 6.7. Fractions (3 ml.) were collected and assayed for P' activity.

13. Ultrafiltration.

Samples were concentrated in an Amicon ultrafiltration apparatus using nitrogen pressure at 45 p.s.i. A PM-10 membrane was used.

14. Protein Determination.

Protein concentrations were measured by the method of Lowry et al. (45). Bovine serum albumin was used as a standard.

15. Sucrose Gradient Centrifugation.

Sucrose velocity gradients were run using a modification of the method of Martin and Ames (44). Linear sucrose gradients were prepared in Lucite mixing chambers. Gradients were made from 5% and 20% (w/v) sucrose in 0.01 M phosphate pH 6.7. These were run in the Beckman L2-65 centrifuge for 16 hours at 32,000 g (max.) in the Type SW 39 rotor. Fractions were collected by puncturing the bottoms of the tubes and were assayed for A_1 and phenol

oxidase activity.

16. Reagents and Buffers.

All reagents were reagent grade. Bovine serum albumin was obtained from Sigma Chemical Co.

Saturated ammonium sulfate solutions were prepared in 0.01 M phosphate pH 6.7 with no adjustment of the final pH.

Phosphate buffers were a mixture of sodium and potassium salts.

RESULTS

Section 1. Assays of Components.

A. General.

In order to devise methods for handling and purification of the various components involved in the activation of Drosophila phenol oxidase, it was necessary to develop quantitative assays for the components. An assay for A_1 has been described elsewhere (2). Assays have been developed for S activator, S, P and P'.

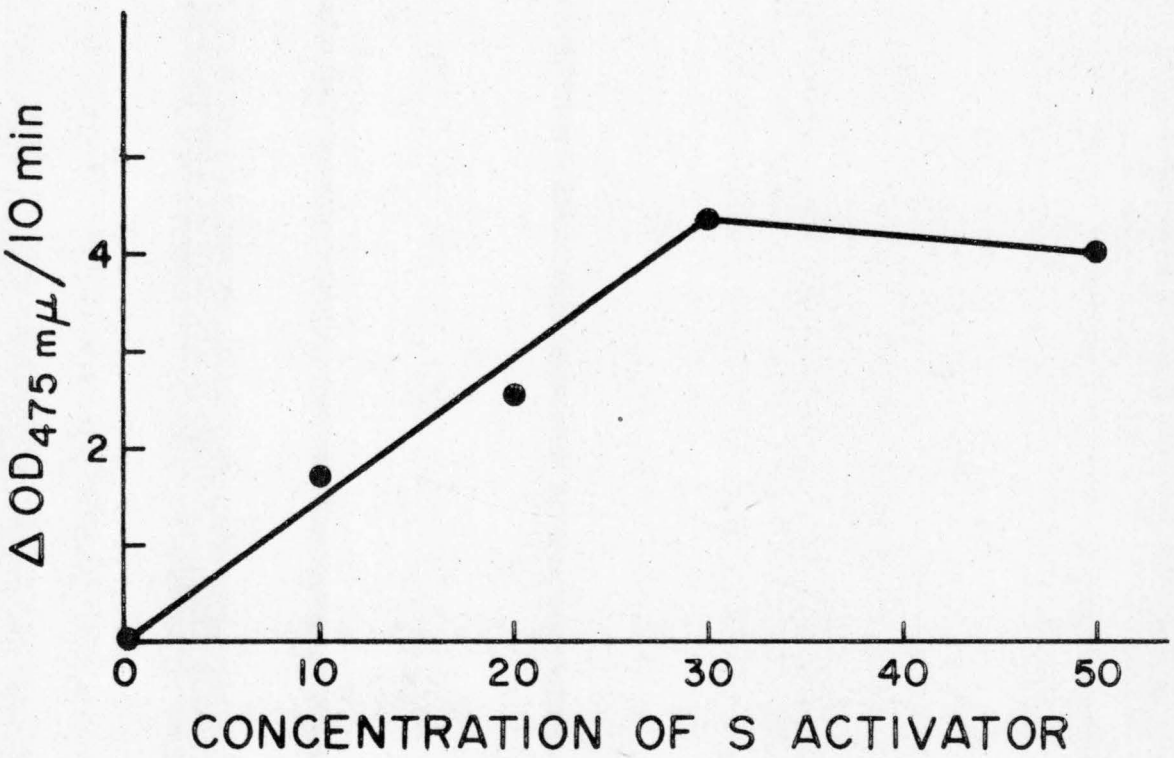
B. S Activator Assay.

The S activator is the component which catalyzes the conversion of the precursor of S, Pre S, to S. S activator was assayed for by incubating an aliquot of the unknown (1 µl. to 50 µl.) with 50 µl. of Pre S (Materials and Methods, section 8) in a total volume of 100 µl. for 60 minutes. The mixture was then assayed for S activity (Results, section 1C). A standard curve for this assay is shown in Figure 2. The assay appears to be fairly linear up to about 4.5 units of activity.

C. S Assay.

S is the component which catalyzes the conversion of P to P'. S activity was assayed for by the use of a slight modification of a method described by Mitchell (34). An aliquot of the unknown (1 µl. to 100 µl.) was incubated

Figure 2. S activator assay. The S activator preparation was a 35-56% ammonium sulfate fraction from 24 hour pupae prepared as described in Materials and Methods, section 9. The ammonium sulfate pellet was dissolved in 5 ml. of buffer per gram of starting material. A series of dilutions was prepared and assayed for S activator as described in the text. The abcissa represents the volume in µl. of S activator in the incubation mixture.

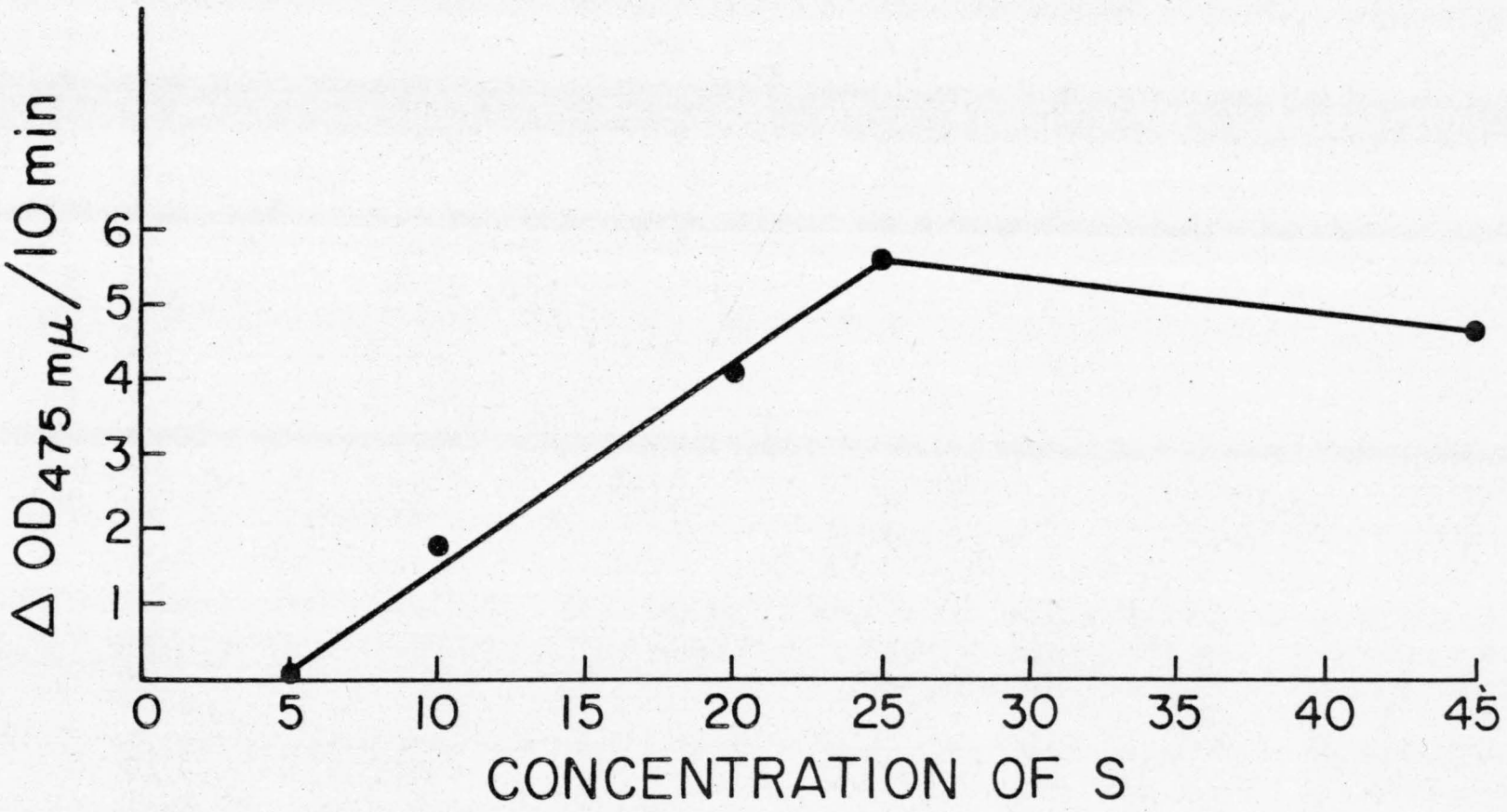

Δ OD475 mμ/10 min
4
2
0
10
20
30
40
50
CONCENTRATION OF S ACTIVATOR

with 25 µl. of H, a 35-56% ammonium sulfate fraction (see Materials and Methods, section 6), in a total volume of 125 µl. for 20 minutes. The phenol oxidase activity in the mixture was then measured. A standard curve for the assay is shown in Figure 3. The curve is linear over about a 5 fold range of S concentration. Since the assay curve intersects the abcissa at a significant S concentration, caution must be observed when working with dilute solutions of S. For example, two fold dilution of a sample which can easily be assayed may bring it out of the useful range of the assay. A unit of S activity is defined as the amount of S which will produce one unit of phenol oxidase activity in the assay. This assay is also sensitive to P' and is therefore only quantitative in the absence of P'.

D. P Assay.

The P component is the precursor of P'. P was assayed for by incubating an aliquot of the unknown (1 µl. to 75 µl.) with 25 µl. of S (prepared through ammonium sulfate fractionation and hydroxylapatite chromatography; Materials and Methods, section 10, A and B) in a total volume of 100 µl. for 60 minutes. An aliquot (25 µl.) of A_1 (Materials and Methods, section 7) was then added to the incubation mixture and the phenol oxidase produced was measured 20 minutes later. A unit of P activity is defined as that amount of P which will produce one unit of phenol oxidase activity in the P assay. A standard curve for the

Figure 3. Standard curve for the S assay. The S was prepared as described in Materials and Methods, section 10. A series of dilutions of the S was prepared and assayed for S activity as described in the text. The abcissa represents the volume in µl. of S in the incubation mixture.


Δ OD475 mμ/10 min
1
2
3
4
5
6
0
5
10
15
20
25
30
35
40
45
CONCENTRATION OF S

assay is shown in Figure 4. The assay is linear up to about 4 units of activity and is sensitive to changes in P over a wide concentration range.

E. P' Assay.

P' is the component formed by the action of S on P. It reacts with A_1 to produce phenol oxidase. P' was assayed for by incubating an aliquot of the unknown (1 µl. to 100 µl.) with 25 µl. of A_1 (Materials and Methods, section 7) in a total volume of 125 µl. for 20 minutes. The mixture was then assayed for phenol oxidase activity. A unit of P' activity is defined as the amount of P' required to produce one unit of phenol oxidase activity in this assay. A standard curve for the assay is shown in Figure 5. The assay is sensitive to changes in P' concentration over a wide range. The curve deviates strongly from linearity at high concentrations of P' but is fairly linear in the range of 0 to 2.5 units.

Section 2. Purification and Characterization of the Components.

A. General.

It was clear that elucidation of the complex series of reactions concerned with phenol oxidase activation would involve purification of the components involved. A primary problem in the handling of phenol oxidase components is the prevention of phenol oxidase activation in extracts.

Figure 4. Standard curve for the P assay. The P protein was prepared from 4 to 8 hour prepupae and partially purified over hydroxylapatite as described in Materials and Methods, section 11. A series of dilutions was prepared and assayed for P activity as described in the text. The abcissa represents the volume in µl. of P in the incubation mixture.

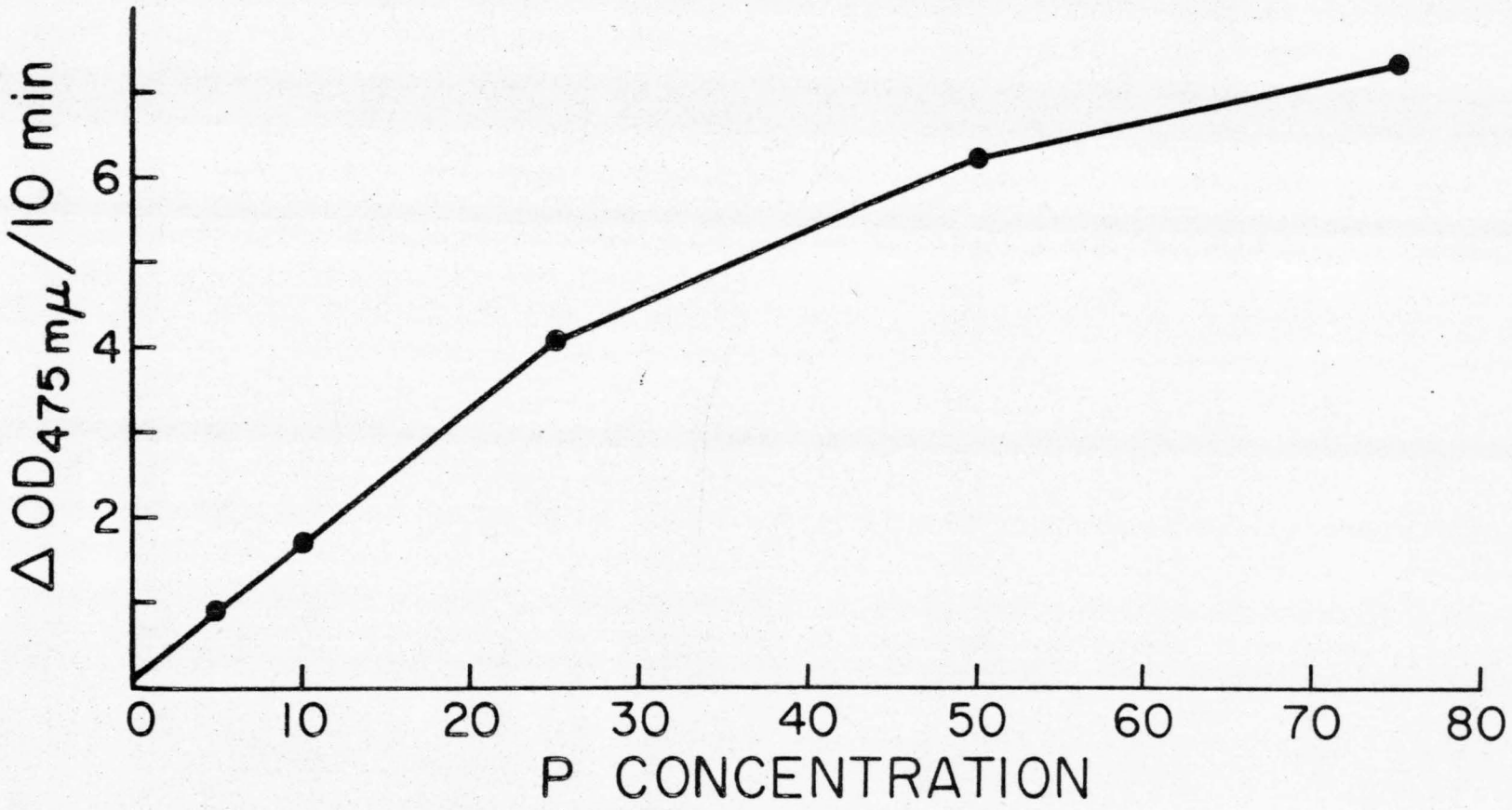
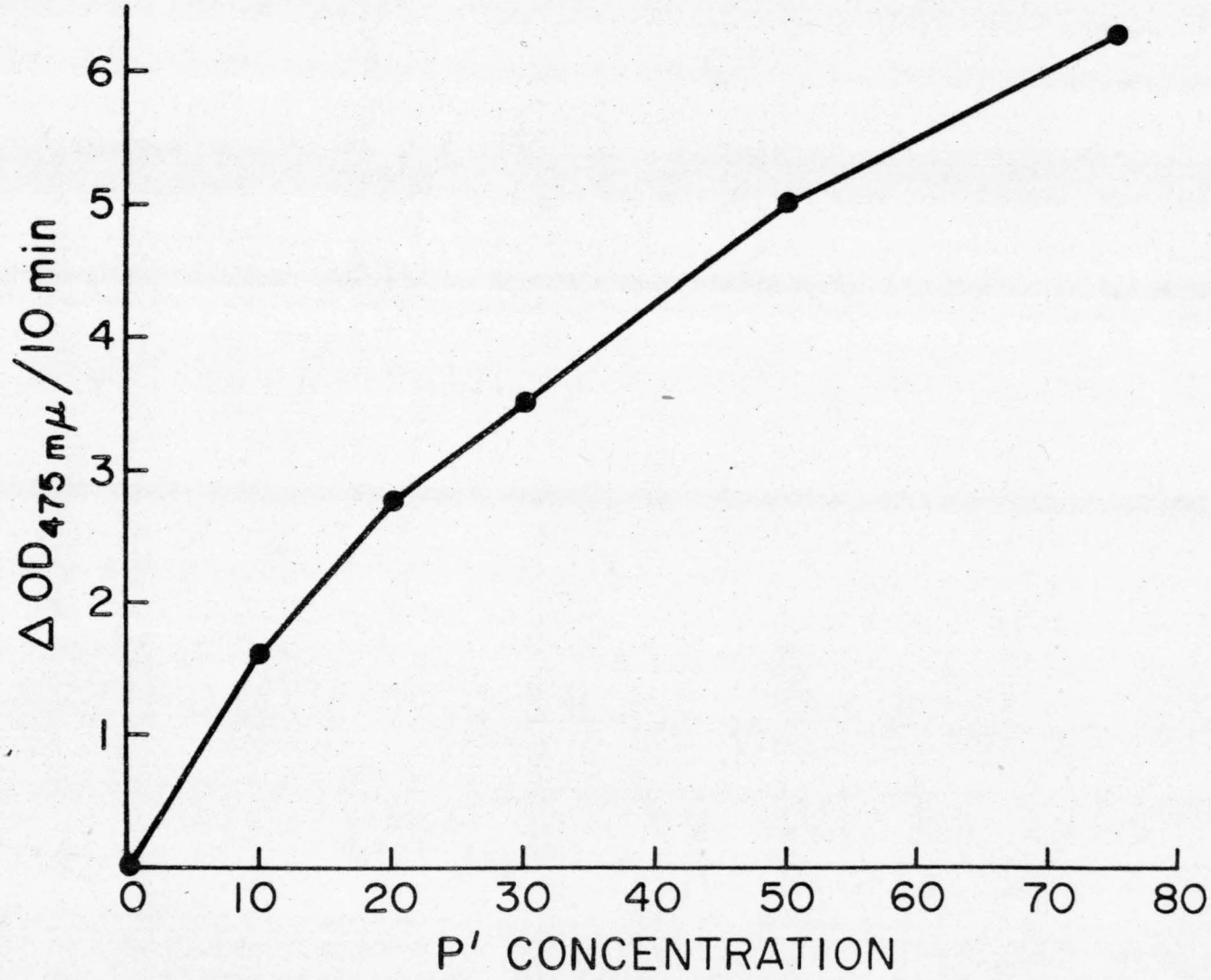

ΔOD475 mμ/10 min
2
4
6
0
10
20
30
40
50
60
70
80
P CONCENTRATION

Figure 5. P' assay. P' was prepared as described in Materials and Methods, section 12, Method I. Dilutions of the P' were made and assayed as described in the text. The abcissa gives the volume of P' in µl. present in the incubation mixture.


ΔOD475 mμ/10 min
6
5
4
3
2
1
0
10
20
30
40
50
60
70
80
P′ CONCENTRATION

Even small amounts of phenol oxidase activity can result in melanization and loss of component activity. Isolation of Pre S and P poses a special problem since these components have a strong tendency to convert to S and P' respectively. Mention is made in the sections devoted to each component of the methods used to prevent activation. This generally involved separation of the components before activation could occur, usually by means of ammonium sulfate fractionation. Use was often made of 4 to 8 hour prepupae since this stage animal lacks S activator activity. This results in the blockage of the conversion of Pre S to S and consequently of the activation of phenol oxidase (see section 3A).

B. S Activator Component.

1. Ammonium Sulfate Fractionation.

S activator was isolated from a 35-56% $(NH_4)_2SO_4$ fraction so that the preparation would not be contaminated with Pre S which is found primarily in the 0-35% $(NH_4)_2SO_4$ fraction. S activator is also present in the 0-35% $(NH_4)_2SO_4$ fraction, however, since the Pre S in this fraction is converted to S without the addition of any other fractions.

2. Hydroxylapatite Chromatography.

S activator was partially purified by chromatography on hydroxylapatite. The behavior of S activator

on this column is seen in Figure 6. One useful aspect of this fractionation is that the S activator is separated from A_1 and A_2 which stick much more tightly to this column than the S activator (2).

C. Pre S Component.

1. Ammonium Sulfate Fractionation.

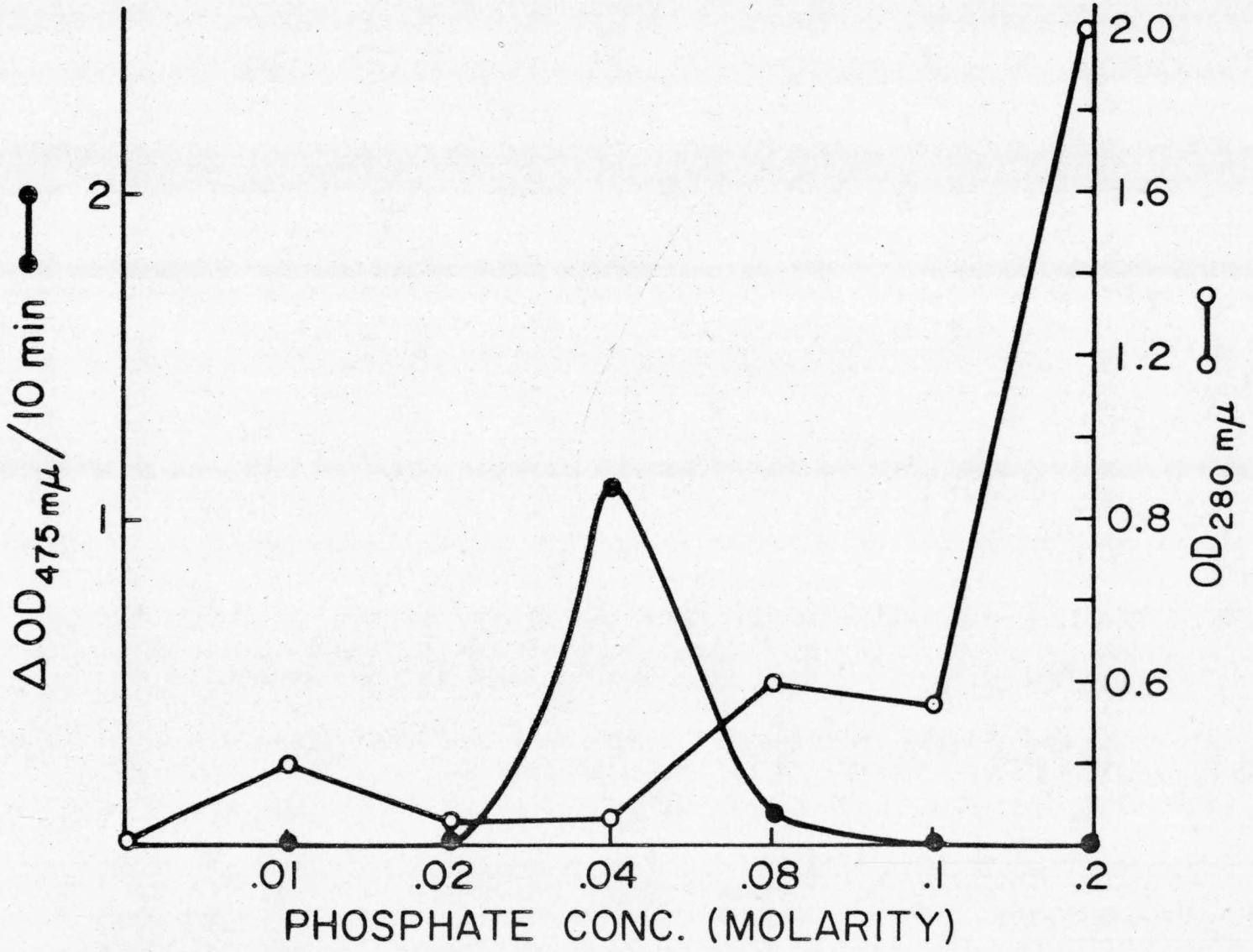
Pre S was prepared as a 0-35% $(NH_4)_2SO_4$ fraction from 4 to 8 hour prepupae. This stage animal lacks the S activator (see section 3A) and as a result Pre S from this stage is relatively stable. Attempts have been made to separate Pre S and the S activator in other stage animals using a variety of techniques but these have not been successful. Attempts to inhibit reversibly the activation of Pre S in solution have also been unsuccessful.

D. S Component.

1. Ammonium Sulfate Fractionation.

In order to prevent phenol oxidase activation, a 0-35% $(NH_4)_2SO_4$ fraction was prepared by grinding 24 hour pupae directly in 35% $(NH_4)_2SO_4$. This blocks activation since the Pre S activity is precipitated at this concentration, while the bulk of the A component activity remains in solution (33). This also prevents the conversion of Pre S to S until the pellet is dissolved. It was found necessary to wash the pellet by resuspending it in 35% $(NH_4)_2SO_4$ and then to extract it with buffer and reprecipitate the Pre S

Figure 6. Hydroxylapatite chromatography of S activator. The S activator preparation was a 35-56% ammonium sulfate fraction from 24 hour pupae prepared as described in Materials and Methods, section 9. 200 µl. of the preparation was applied to a one ml. hydroxylapatite column equilibrated with .01 M phosphate pH 6.7. The column was eluted with a step gradient, 2 ml. each of 0.02 M, 0.04 M, 0.08 M, 0.1 M and 0.2 M phosphate pH 6.7. Aliquots (50 µl.) of the fractions were assayed for S activator and the $OD_{280\ m\mu}$ was measured.

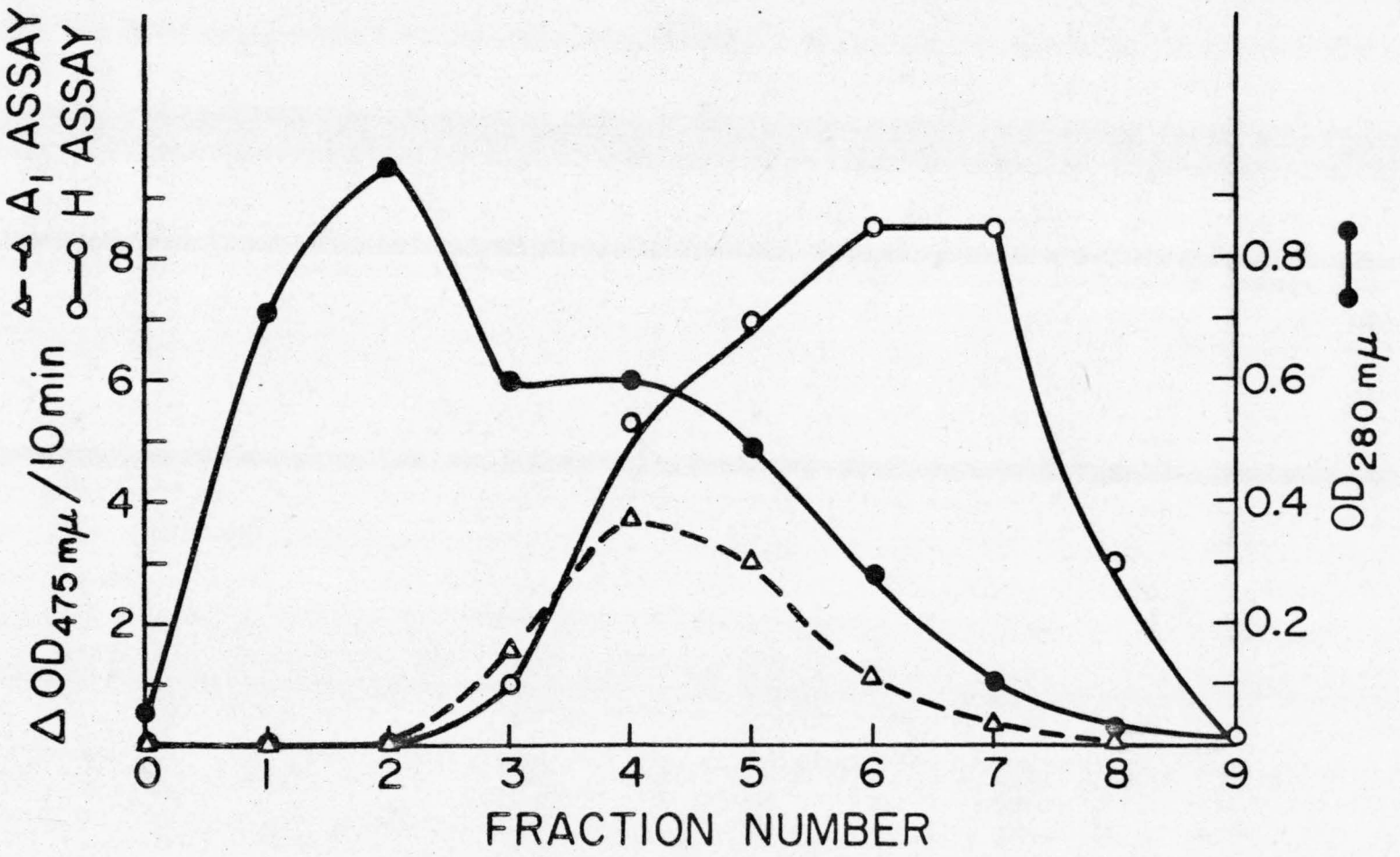

Δ OD 475 mμ / 10 min
2
OD 280 mμ
2.0
1.6
1.2
0.8
0.6
.01
.02
.04
.08
.1
.2
PHOSPHATE CONC. (MOLARITY)

activity by bringing the $(NH_4)_2SO_4$ concentration back to 35%. This step was necessary to free the pellet of residual A components. If this was not done, phenol oxidase activation was observed when the pellet was dissolved. When the pellet was finally dissolved it was allowed to sit in the cold for at least 3 hours to allow complete conversion of the Pre S to S. This fraction was found to contain both S and also P', the component which is produced by the action of S on P. The 10 g. of starting material contained approximately 1500 mg. of soluble protein. Approximately 25 mg. of protein were recovered in the crude S fraction.

2. Hydroxylapatite Chromatography.

Further fractionation was required to separate the S and P' activities. Chromatography on hydroxylapatite was used for this purpose and the results of such an experiment are shown in Figure 7. The S activity was significantly retarded on the column with respect to the P' activity and although a complete separation was not obtained it was possible to obtain an S fraction free of P' by pooling tubes from the trailing edge of the peak. This fraction did not react with A_1 over a wide concentration range and was therefore considered free of P' which does react with A_1. The absence of P' also indicated the absence of P since P is rapidly converted into P' in the presence of S (see section 3B). A_1 was also considered to be absent

Figure 7. Hydroxylapatite chromatography of crude S. The sample was 5 ml. of crude S prepared from 10 g. of 24 hour pupae as described in Materials and Methods, section 10. The column was eluted with a step gradient of phosphate buffer. Fractions 1 to 5 were eluted with 0.1 M phosphate pH 6.3. Fractions 6 to 9 were eluted with 0.02 M phosphate pH 6.7. 2 ml. fractions were collected. 25 µl. aliquots were assayed for either S or P' with H or A_1 respectively.


Δ OD475 mμ/10 min
A1 ASSAY
H ASSAY
OD280 mμ
FRACTION NUMBER
0
1
2
3
4
5
6
7
8
9
2
4
6
8
0.2
0.4
0.6
0.8

from the preparation since no phenol oxidase activity was produced when P' was added to the fraction. If A_1 had been present it would have reacted with the P' to produce phenol oxidase (see section 3C). The specific activity of S prepared in this manner was 10,000 units/mg. The starting material contained 25 mg. of protein and 250 µg. were recovered in the S fraction. Yields or fold purification cannot be calculated for either this fractionation or the previous one since the presence of P' interferes with the quantitative assay for the S component.

E. P Component.

1. General.

A major difficulty in the preparation of the P component was the prevention of its conversion to P' by S. For this reason 4 to 8 hour prepupae were used in the preparation of P since the lack of S activator activity in these animals blocks the production of S and hence the conversion of P to P'.

2. Ammonium Sulfate Fractionation.

P was prepared as a 36-50% fraction from 4 to 8 hour prepupae. It proved to be relatively stable in this form but trace amounts of S in the preparation sometimes caused the production of P' on prolonged standing (24 hours).

3. Hydroxylapatite Chromatography.

In order to separate P from any trace amounts of S and also from A components present in the 36-50% fraction, this fraction was applied to a hydroxylapatite column immediately on resuspension of the 36-50% pellet. The elution behavior of P on this column is shown in Figure 8. The yield and fold purification in this step are shown in Table 1. The P activity was approximately 12 fold purified over the ammonium sulfate fraction. The P prepared in this manner was considered to be free of S activity since no conversion to P' was observed on prolonged incubation (24 hours). The preparation was also considered to be free of A_1 since no phenol oxidase was produced on addition of P' which would have reacted with any A_1 present to produce phenol oxidase.

4. Bio-Gel P-60 Chromatography.

P which had been prepared through ammonium sulfate fractionation was chromatographed on a P-60 column. The results are shown in Figure 9. The bulk of the P activity is excluded on this column. The tail on the P peak is believed to be due to P' contaminating the preparation. This column is of no particular value in the purification of P but it does suggest that the minimum molecular weight of P is 60,000 daltons. This result is of interest in discussing possible mechanisms of the activation reaction.

Figure 8. Hydroxylapatite chromatography of P. The sample was 3 ml. of a 35-50% ammonium sulfate fraction prepared from 2 g. of 4 to 8 hour prepupae as described in Materials and Methods, section 11. The column had a 5 ml. bed volume and was equilibrated with 0.01 M phosphate pH 6.7. The column was eluted with a step gradient and 2 ml. fractions were collected. Fractions 1 to 5 were eluted with 0.05 M phosphate pH 6.7. Fractions 6 to 10 were eluted with 0.1 M phosphate pH 6.7. The $OD_{280\ m\mu}$ was measured and 25 µl. aliquots were assayed for P activity.

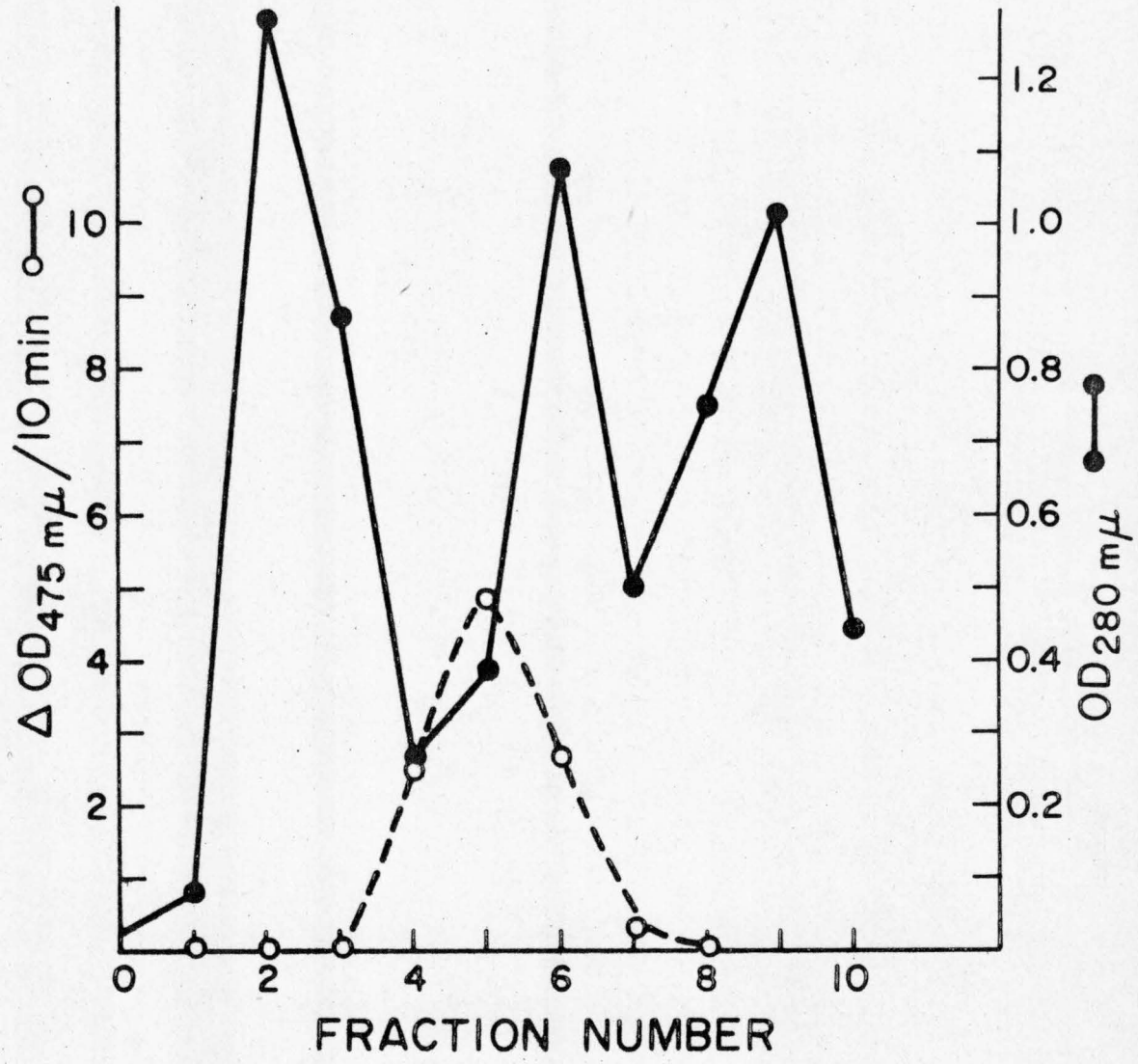
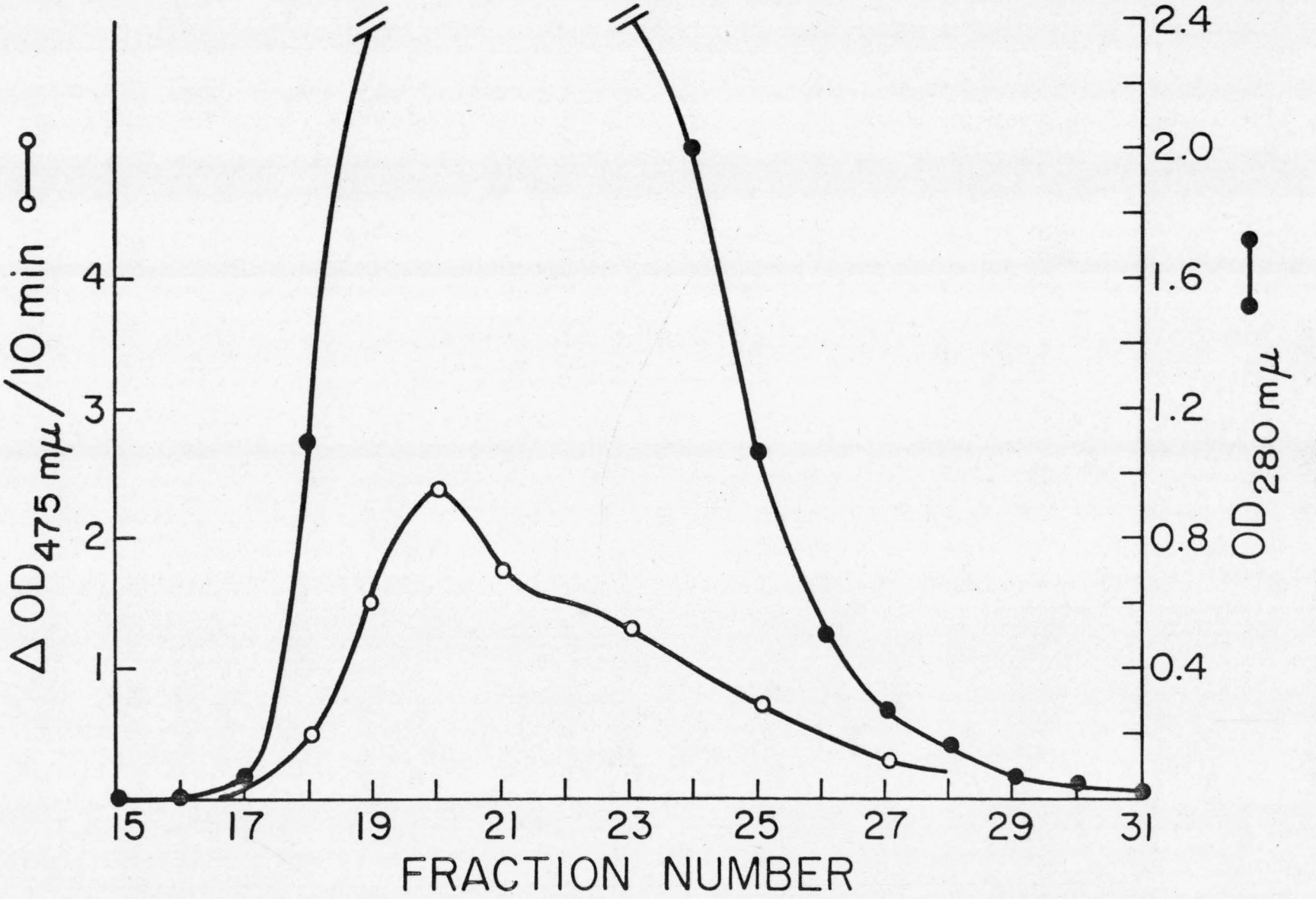

Δ OD475 mμ/10 min
OD280 mμ
FRACTION NUMBER
0
2
4
6
8
10
1.2
1.0
0.8
0.6
0.4
0.2

Table 1.

Partial Purification of P.

Fraction	Protein (mg.)	Activity (units)	Specific Activity (units/mg.)	Yield %
Ammonium sulfate	48	1200	25	-
Hydroxylapatite	3	960	320	80

Figure 9. Bio-Gel P-60 chromatography of P. The starting sample was 3 ml. of a 35-50% ammonium sulfate fraction prepared from 2 g. of 4 to 8 hour pre-pupae as described in Materials and Methods, section 11. The column was 2.5 x 80 cm. The elution buffer was 0.01 M phosphate pH 6.7. The flow rate was 8.5 ml. per hour. 4.5 ml. fractions were collected. The $OD_{280\ m\mu}$ was monitored and 50 µl. aliquots were assayed for P activity.


Δ OD475 mμ/10 min
OD280 mμ
FRACTION NUMBER
15
17
19
21
23
25
27
29
31
1
2
3
4
0.4
0.8
1.2
1.6
2.0
2.4

F. P' Component.

1. Ammonium Sulfate Fractionation.

The primary problem in the preparation of P' was to ensure its separation from the A components before phenol oxidase activation could occur. Since the crude S preparation (described in section 2D part 1 above) contained large quantities of P' and no A_1 the same ammonium sulfate fractionation was used for the preparation of P'. The specific activity of P' in this fraction is shown in Table 2.

2. Bio-Gel P-60 Chromatography.

P' prepared through ammonium sulfate fractionation was chromatographed on a Bio-Gel P-60 column. The results are shown in Figure 10. The P' activity was separated from the bulk of the protein eluting from this column. Table 2 shows the fold purification and yields for this step. The Kav. of 0.35 indicates a molecular weight of about 30,000 daltons (43).

3. Bio-Gel P-30 Chromatography.

Gel filtration on P-30 has not been used in an integrated purification scheme for P' but it is a potentially useful step. P' was prepared through ammonium sulfate fractionation and applied to a P-30 column. The results are shown in Figure 11. P' was slightly included

Figure 10. Bio-Gel P-60 chromatography of P'. The sample was 5 ml. of P' prepared through ammonium sulfate fractionation as described in Materials and Methods, section 12, Method IIA. The column was 2.5 x 80 cm. and was eluted with 0.01 M phosphate pH 6.7 at a flow rate of 8.5 ml./hour. Fractions (4.5 ml.) were collected and 100 µl. aliquots were assayed for P' activity. The $OD_{280\ m\mu}$ was measured.

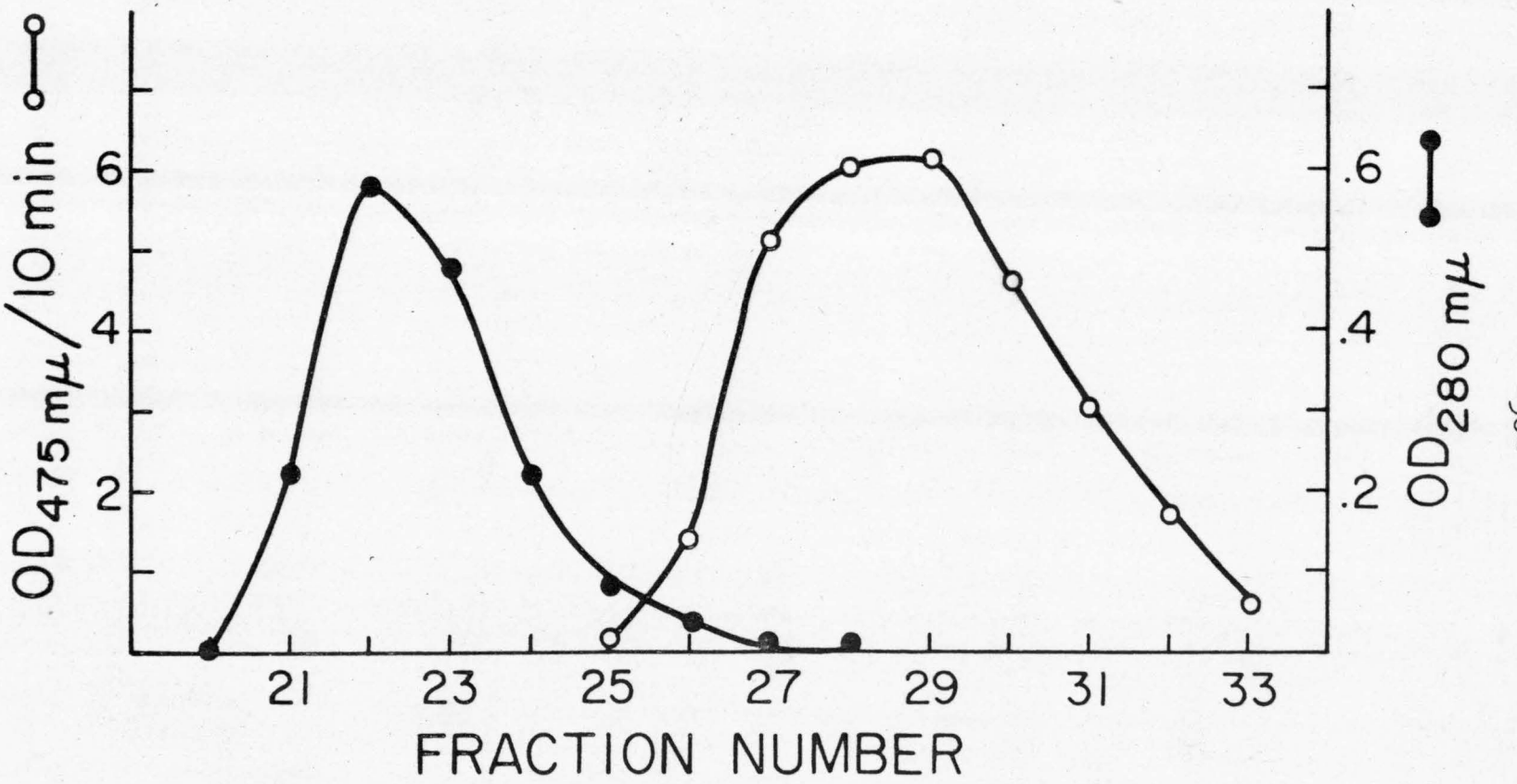
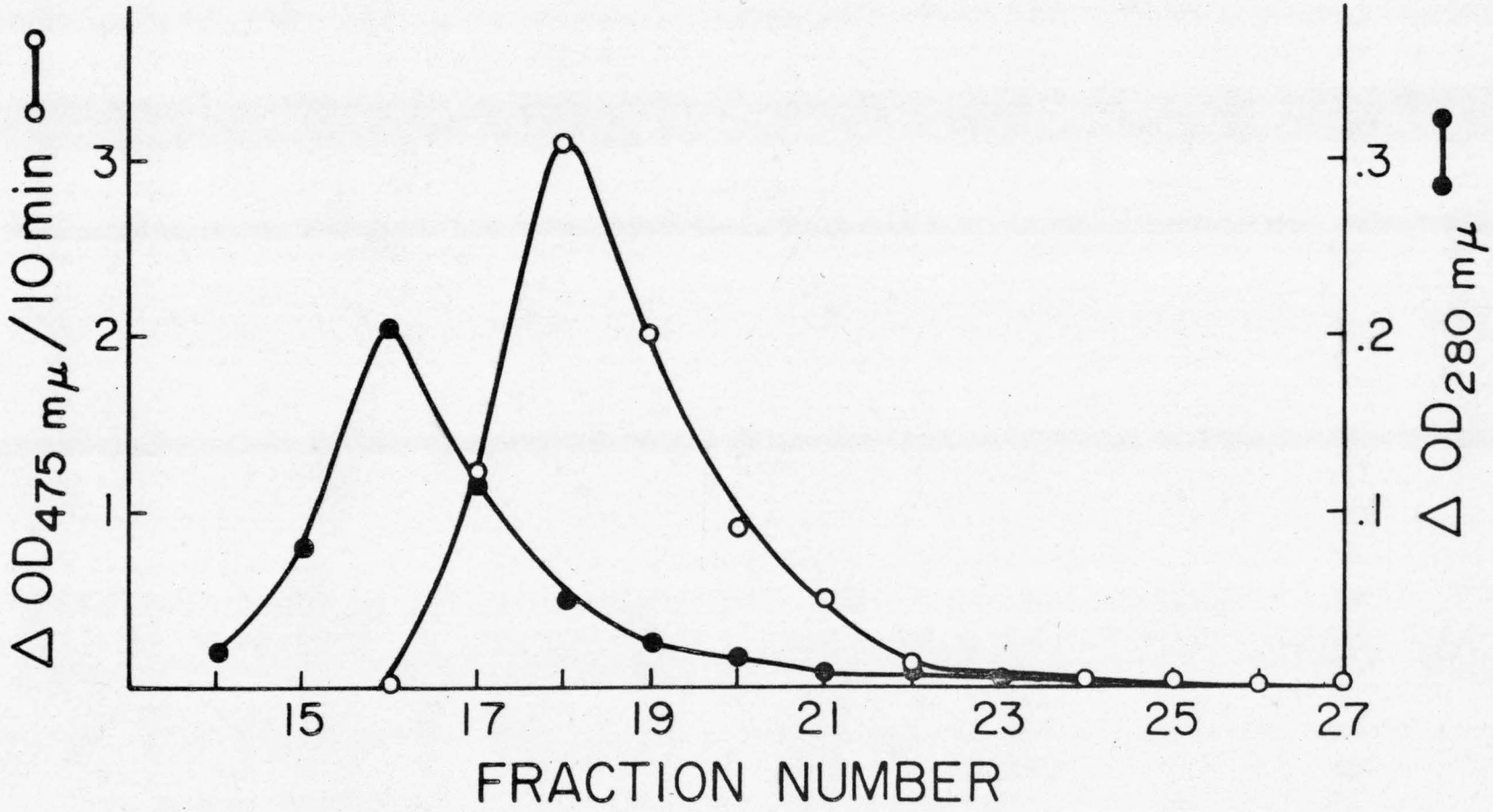

OD475 mμ/10 min
2
4
6
.2
.4
.6
OD280 mμ
21
23
25
27
29
31
33
FRACTION NUMBER

Figure 11. Bio-Gel P-30 chromatography of P'. The sample was 2 ml. of P' prepared as described in Materials and Methods, section 12, Method IIA. The column was 2.5 x 35 cm. Fractions (3 ml.) were collected and 100 µl. aliquots were assayed for P' activity. The $OD_{280\ m\mu}$ of the fractions was measured.


Δ OD475 mμ / 10 min
Δ OD280 mμ
1
2
3
.1
.2
.3
15
17
19
21
23
25
27
FRACTION NUMBER

with a Kav. of 0.15. This again would indicate a molecular weight in the neighborhood of 30,000 daltons (43).

4. Hydroxylapatite Chromatography.

P' which had been purified through ammonium sulfate fractionation and P-60 chromatography was chromatographed on a hydroxylapatite column. The percentage yields, fold purification and the specific activity of the eluted P' are shown in Table 2.

G. A_1 Component.

1. Ammonium Sulfate Fractionation.

A_1 has been purified to homogeneity (2). The purification is a time consuming task however and for this reason an ammonium sulfate fractionation scheme was devised to prepare A_1 which was suitable for use in the P and P' assays. Experiments demonstrating the suitability of A_1 prepared in this way are summarized in Table 3. The lack of reaction with S indicates the absence of P in the preparation.

Section 3. Reactions of the Components.

A. Reaction of Pre S and S Activator.

The existence of a Pre S - S activator system was suggested following Mitchell's observation that S activity is latent and appears in homogenates on incubation at

Table 2.

Partial Purification of P'.

Fraction	Protein (mg.)	Activity (units)	Specific Activity (units/mg.)	Yield % Step	Yield % Total	Fold Purification Step	Fold Purification Total
Ammonium sulfate	27	1600	59	-	-	-	-
P-60	1	560	560	35	35	9.5	9.5
Hydroxyl-apatite	0.05	210	4,200	37	13	7.5	71

Table 3.

Reactions of Partially Purified A_1 With S.

Reaction	S Fraction Vol.(µl.)	S Fraction Type	A_1 Fraction Vol.(µl.)	A_1 Fraction Type	Buffer (µl.)	Phenol Oxidase (units)
1.	25	Purified[1]	25	A_1 [3]	75	0
2.	25	Purified	25	H [4]	75	9.5
3.	25	Crude[2]	25	A_1	75	9.5
4.	0	-	100	A_1	25	0.15
5.	0	-	25	A_1	100	0

[1]Materials and Methods, section 10B

[2]Materials and Methods, section 10A

[3]Materials and Methods, section 7

[4]Materials and Methods, section 6

0°C. (35). The results of an experiment confirming this observation are shown in Figure 12.

The hypothesis that a two component system was necessary for the production of S activity was confirmed by mixing experiments using extracts of 4 to 8 hour prepupae and 24 hour pupae. Mitchell had previously made the observation that S activity was lacking in extracts of 4 to 8 hour prepupae(35) and he made the suggestion that this lack occurred because the precursor of S was not converted into S in these animals. This was confirmed by the results of the experiment described in Figure 13. A 0-35% $(NH_4)_2SO_4$ fraction from 4 to 8 hour prepupae was mixed with a 35-56% fraction from 24 hour pupae and the appearance of S activity was observed in the homogenate. This experiment showed that potential S activity (Pre S) does exist in 4 to 8 hour prepupae and that there is a substance (S activator) in 24 hour pupae which can cause the conversion of this potential activity into active S.

B. Reaction of S and P.

1. General.

Previous experiments had suggested the possibility that S and P react to form a product, which was named P', which in turn reacts with A_1 to produce phenol oxidase. A series of mixing experiments using variable concentrations of S and P incubated together for a variable

Figure 12. Activation of S. Crude S was prepared as described in Materials and Methods, section 10A. 25 µl. aliquots were withdrawn at varying times following resuspension of the ammonium sulfate pellet and assayed for S activity. The abcissa represents time following resuspension of the pellet.

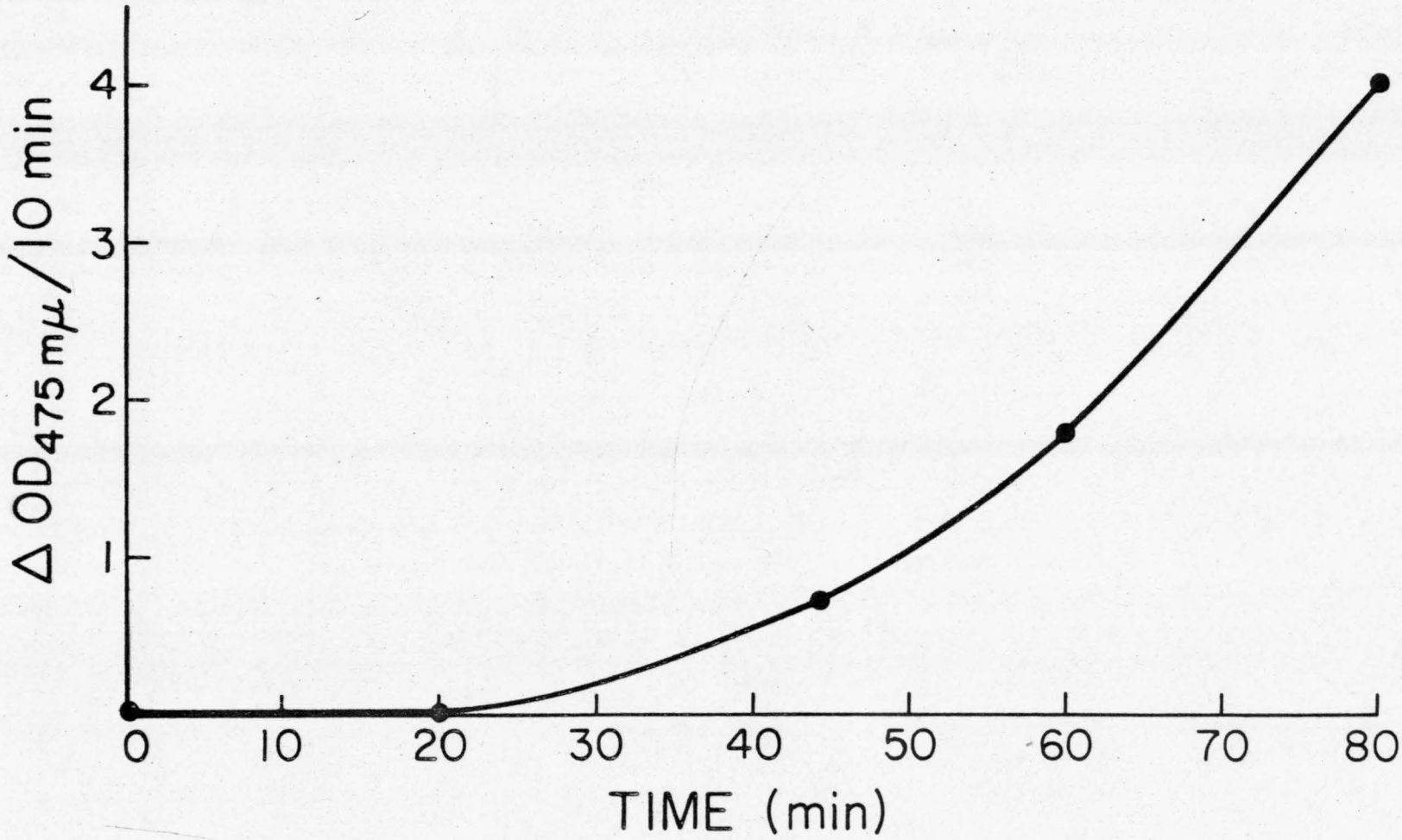
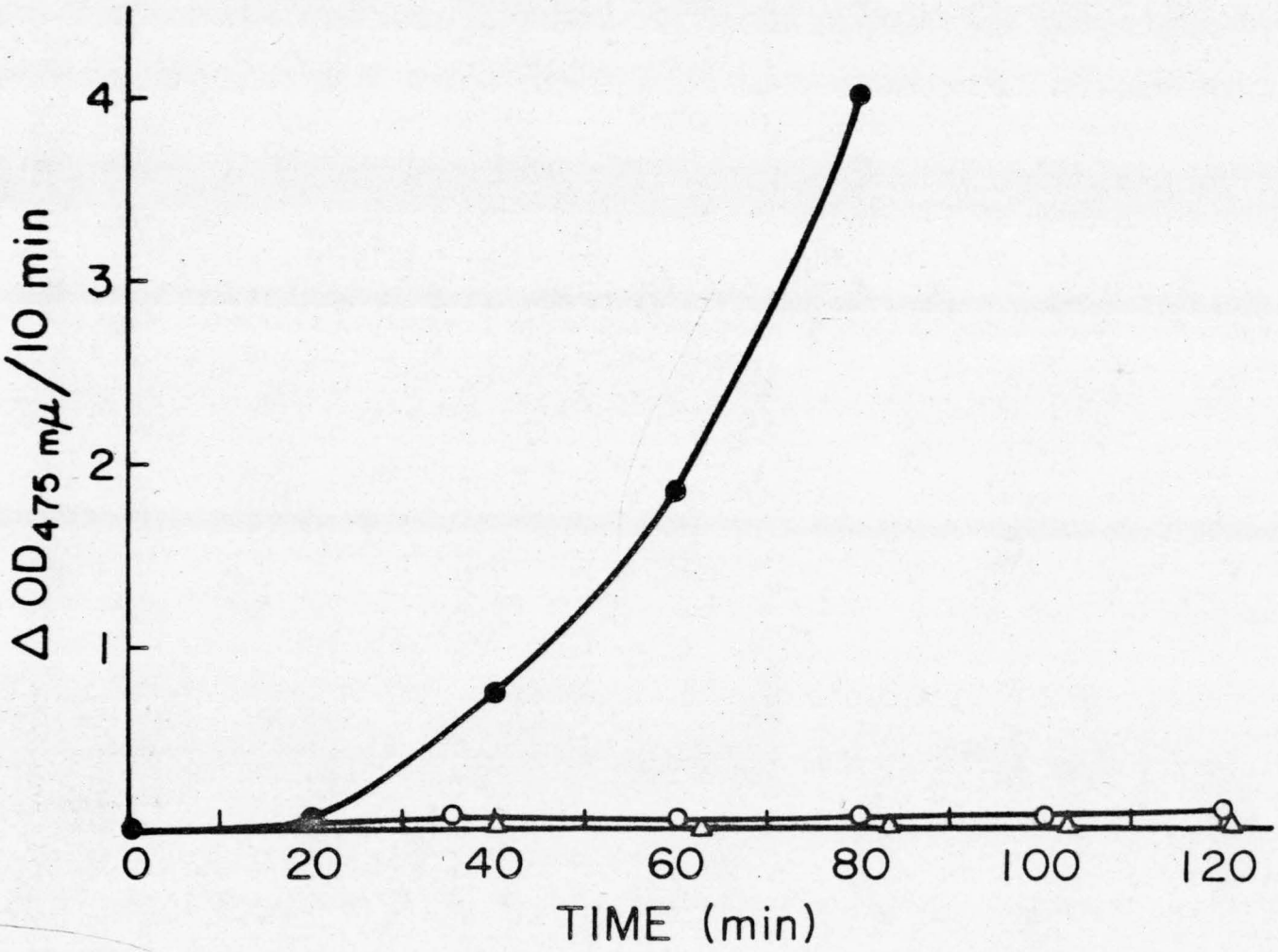

Δ OD475 mμ/10 min
4
3
2
1
0
10
20
30
40
50
60
70
80
TIME (min)

Figure 13. Activation of Pre S with a 35-56% ammonium sulfate fraction from 24 hour pupae. A 0-35% $(NH_4)_2SO_4$ fraction from 4 to 8 hour prepupae and a 35-56% $(NH_4)_2SO_4$ fraction from 24 hour pupae were prepared as described in Materials and Methods, sections 8 and 9. A 1:1 mixture of the two fractions was made and 25 µl. aliquots were withdrawn and assayed for S activity at varying times. In addition, the 0-35% and 35-56% fractions were each diluted 1:1 with buffer and aliquots were withdrawn at varying times for the assay of S activity. The abcissa represents time following the mixing of the two fractions. The closed circles represent the mixture of the two fractions, the open circles represent the 0-35% fraction alone and the triangles represent the 35-56% fraction.


Δ OD475 mμ/10 min
4
3
2
1
0
20
40
60
80
100
120
TIME (min)

time were conducted in an attempt to elucidate the nature of this reaction. The S used in the following experiments was prepared as described in Materials and Methods, section 10 and the P was prepared as described in section 11, parts A and B.

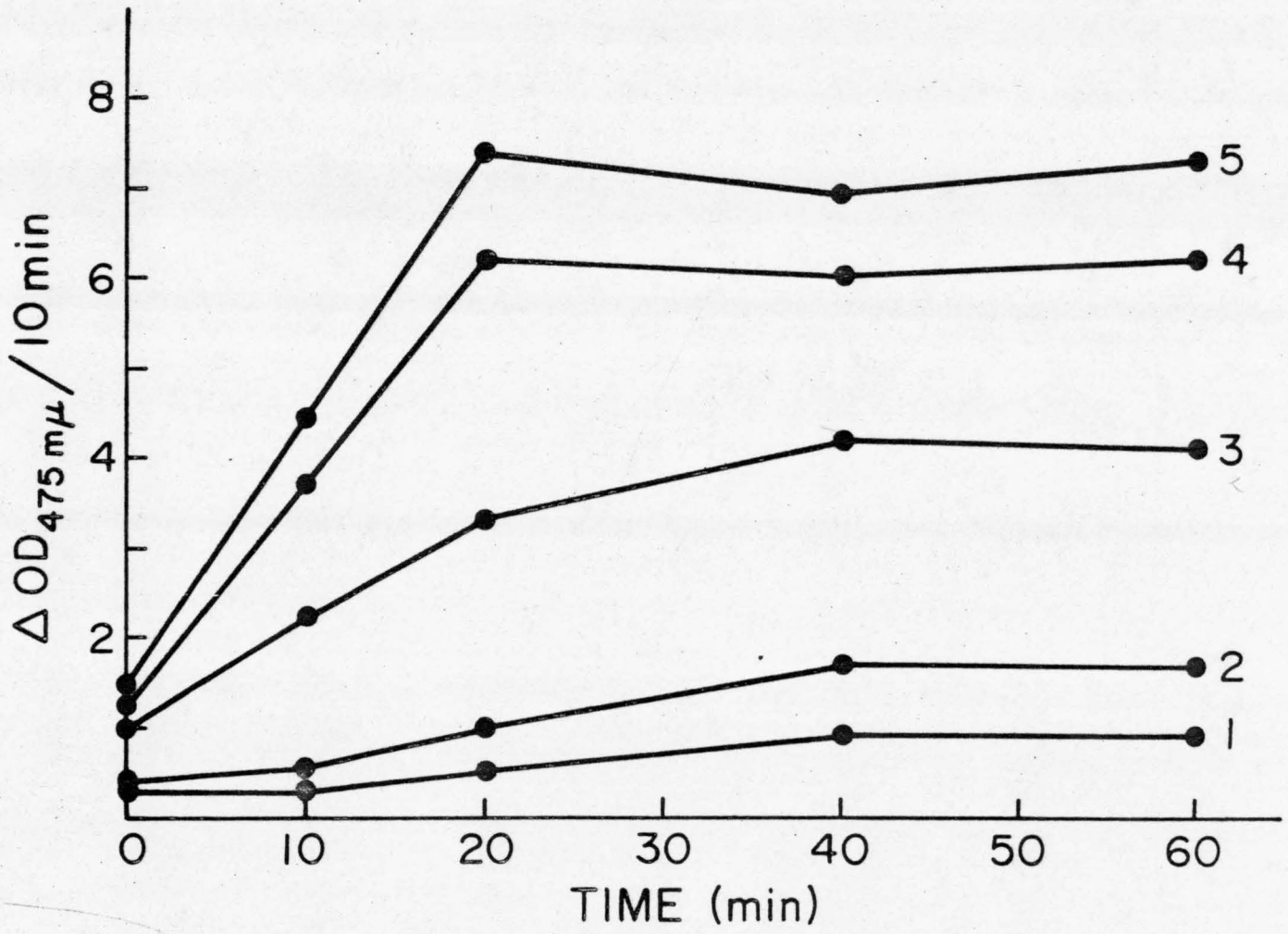
2. Excess S and Variable P.

In order to determine whether P can be limiting in low concentrations in the S and P reaction, experiments were conducted using high constant concentrations of S and low variable concentrations of P. A series of incubation mixtures were set up, each containing a high constant concentration of S. Variable concentrations of P were added to each tube and aliquots were withdrawn at varying times and assayed for P' activity with A_1. The results of such an experiment are shown in Figure 14. This experiment confirmed the observation that S and P react to form P' activity. This experiment also shows that P can become limiting in the reaction. After 20 minutes at high P concentrations and 40 minutes at lower concentrations, no further increase of P' activity occurred and the final levels of activity reached were dependent on the concentration of P. In order to show that P was in fact limiting when the curve levelled off, a mixture of excess S and low P was allowed to react to equilibrium, following which additional P was added and indeed additional P' activity was produced.

Figure 14. P concentration curves. P, S and A_1 were prepared as described in the text.

Curve 1: each point represents 5 µl. of P incubated with 25 µl. of S in a total volume of 100 ul. for the time indicated on the abcissa. Following this time 25 µl. of A_1 were added for 20 minutes and the phenol oxidase in the incubation mixture was assayed.

Curves 2 to 5 represent the same experiment using 10 µl., 25 µl., 50 µl., and 75 µl. of P per point, respectively.


8
6
4
2
ΔOD475mμ / 10 min
0
10
20
30
40
50
60
TIME (min)
5
4
3
2
1

The results of this experiment are summarized in Figure 15.

3. Excess P and Variable S.

In order to determine whether S can be limiting in low concentrations in the S and P reaction, experiments were conducted using high constant concentrations of P and low variable concentrations of S. A series of incubation mixtures were set up, each containing a high constant concentration of P. Variable concentrations of S were added to each tube and aliquots were withdrawn at varying times and assayed for P' activity by incubation with A_1. The results of such an experiment are shown in Figure 16. This experiment indicated that, in contrast to P, there was no evidence that S could become limiting in the reaction. The highest concentration of S did level off but this was due to limiting A_1 in the P' assay. With the lower concentrations of S, P' activity continued to increase, even following 90 minutes of incubation. In contrast, even the lowest concentrations of P reached equilibrium in 40 minutes. It would thus appear that S acts in a catalytic fashion on P to produce P'. The results of this experiment also suggested an alternative assay procedure for the S component. It appeared that the amount of P' formed at 60 minutes in an incubation mixture containing excess P and variable concentrations of S was proportional to the S concentration. The results of an experiment confirming this

Figure 15. Limiting nature of P in the P plus S reaction. P, S and A_1 were prepared as described in the text.

1. Closed circles. Each point represents 10 µl. of P and 25 µl. of S in 100 µl. total volume incubated for the time indicated on the abcissa followed by a 20 minute incubation with 25 µl. of A_1 and an assay of the phenol oxidase produced. At the arrow 50 µl. of additional P was added.

2. Open circles. A parallel experiment in which no P was added.

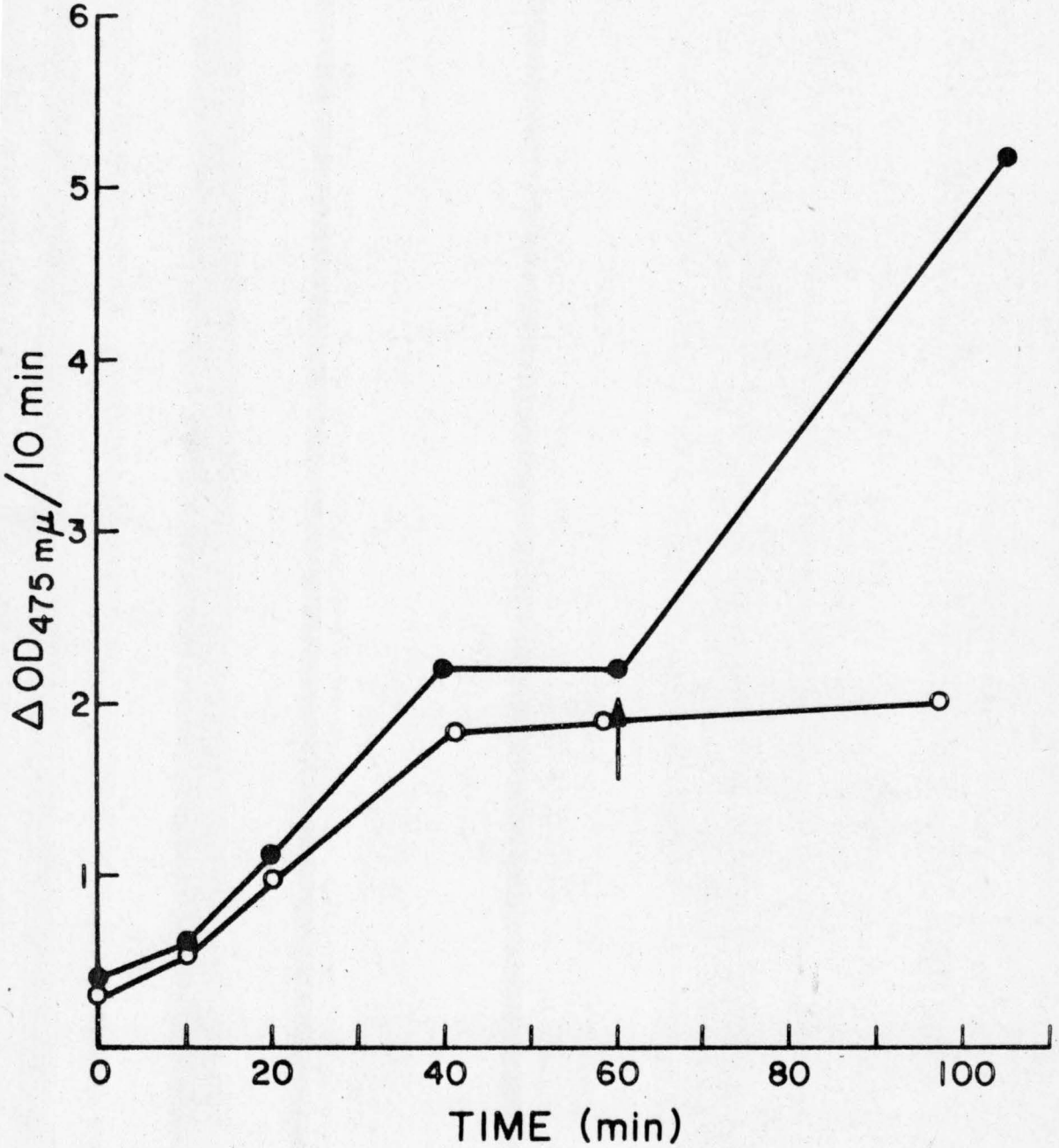
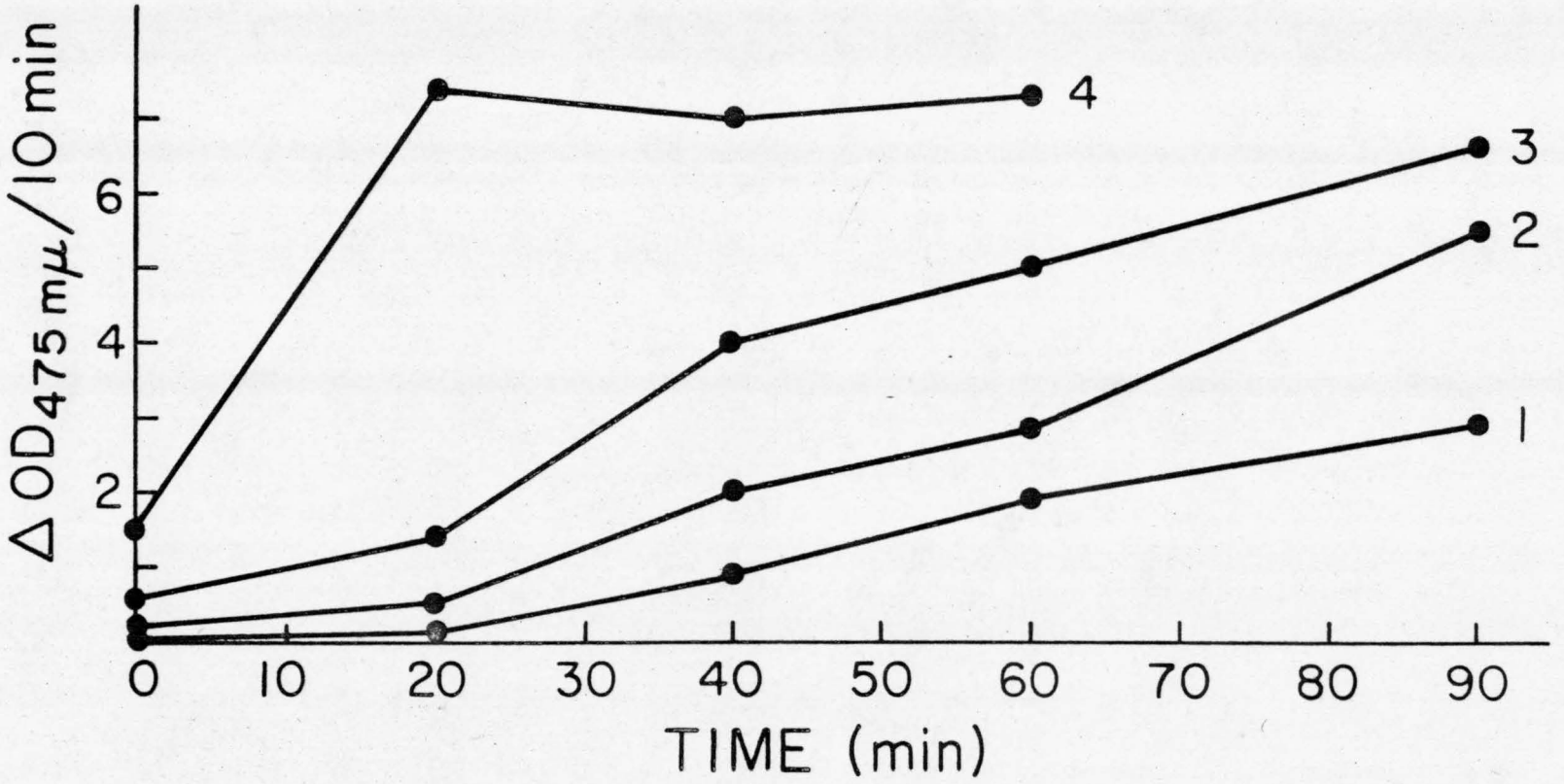

6
5
4
3
2
1
ΔOD475 mμ/10 min
0
20
40
60
80
100
TIME (min)

Figure 16. S concentration curves. P, S and A_1 were prepared as described in the text.

Curve 1: each point represents 5 µl. of S incubated with 75 µl. of P in a total volume of 100 µl. for the time indicated on the abcissa. Following this time 25 µl. of A_1 were added for 20 minutes and the phenol oxidase in the incubation mixture was assayed.

Curves 2, 3 and 4 represent the same experiment using 10 µl., 15 µl. and 25 µl. of S per point respectively.


Δ OD475 mμ/10 min
2
4
6
0
10
20
30
40
50
60
70
80
90
TIME (min)
1
2
3
4

observation are shown in Figure 17. This assay is fairly linear in the range from 1 to 4 units and is sensitive to changes in S concentration over a wider range than the H assay (see Results, section 1C).

C. Reaction of P' and A_1.

1. General

Previous experiments had indicated that P' and A_1 react to produce phenol oxidase. In an attempt to elucidate the nature of this reaction, a series of mixing experiments were conducted using variable concentrations of P' and A_1. Unless indicated otherwise the P' used in the following experiments was prepared as described in Materials and Methods, section 12, Method I and the A_1 was prepared as described in section 7.

2. Excess A_1 and Variable P'.

In an attempt to determine the effects of variations in P' concentrations on the rate of the reaction and on the final levels of activity produced, experiments were conducted using high constant concentrations of A_1 and variable concentrations of P'. A series of incubation mixtures were set up, each containing a high constant concentration of A_1. Variable concentrations of P' were added to the tubes and aliquots were withdrawn at varying times and assayed for phenol oxidase activity. The results of such an experiment are shown in Figure 18. The reaction of P'

Figure 17. Alternative assay for S. S was prepared as described in Materials and Methods, section 10. Aliquots (5-50 µl.) of S were incubated with 50 µl. of P (Materials and Methods, section 11) for 60 minutes. A_1 (25 µl., Materials and Methods, section 7) was added and the phenol oxidase activity was measured 20 minutes later. The abcissa represents the volume in µl. of the S in the incubation mixture.

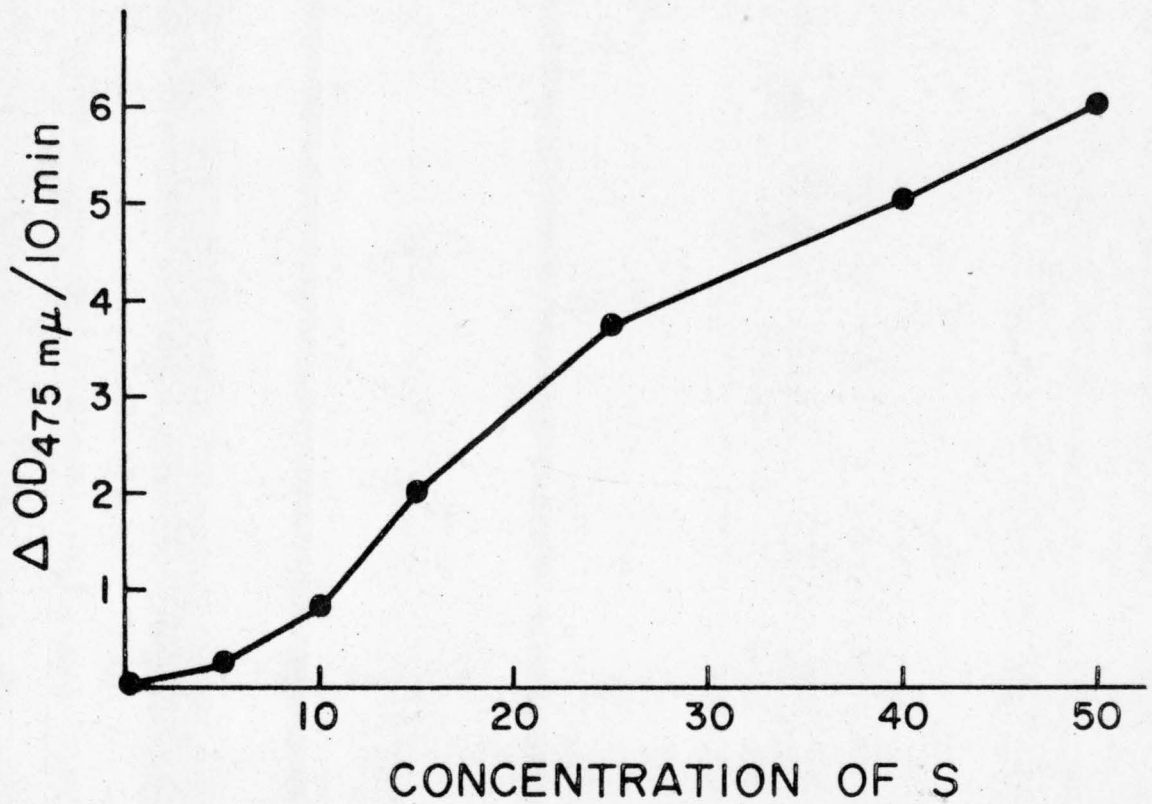
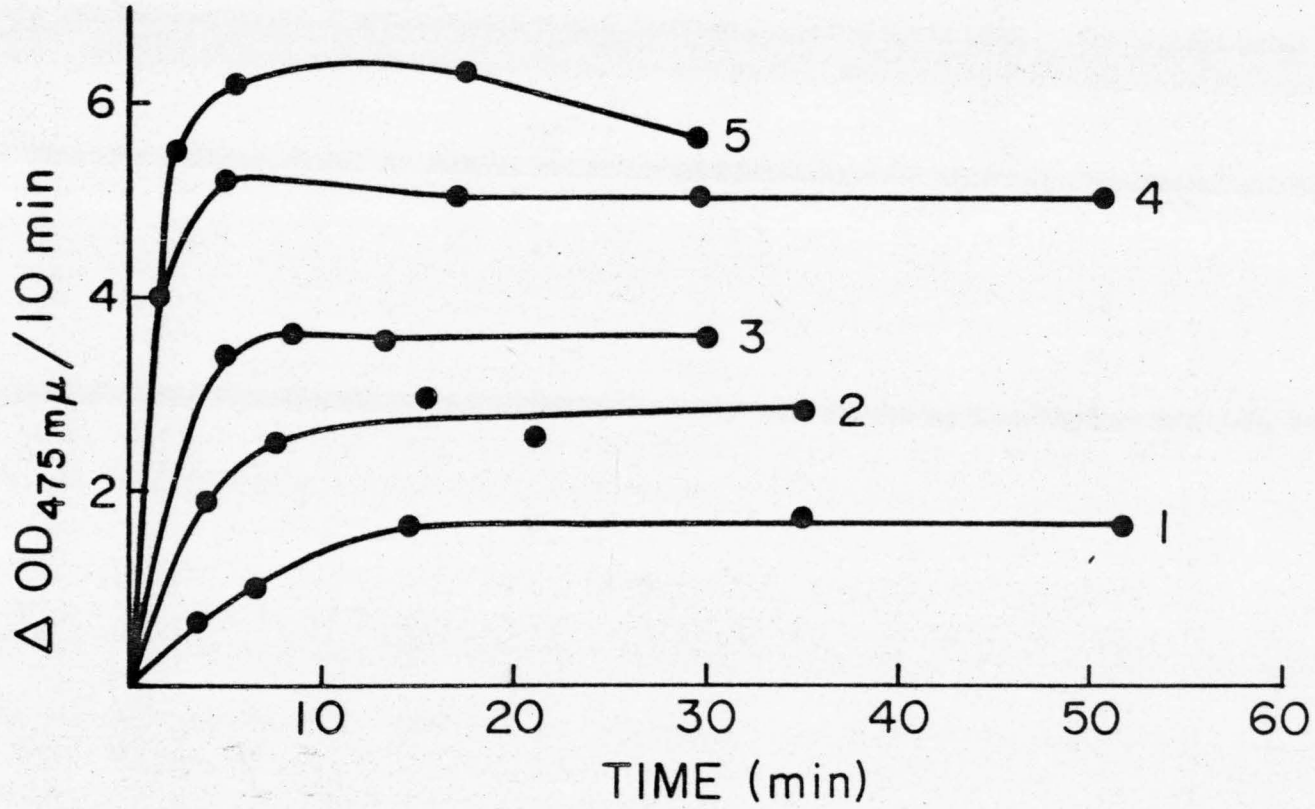

Δ OD475 mμ/10 min
1
2
3
4
5
6
10
20
30
40
50
CONCENTRATION OF S

Figure 18. P' concentration curve. P' and A_1 were prepared as described in the text.

Curve 1: each point represents 10 µl. of P' incubated with 25 µl. of crude A_1 in a total volume of 100 µl. for the time indicated on the abcissa followed by a phenol oxidase assay.

Curves 2 to 5 represent the same experiment using 20 µl., 30 µl., 50 µl. and 75 µl. of P' per point respectively.


Δ OD475mμ/10 min
6
4
2
5
4
3
2
1
10
20
30
40
50
60
TIME (min)

with A_1 occurred quite rapidly, reaching completion in 5 minutes or less at the higher concentrations and in 15 minutes at the lower concentrations. A point of considerable interest was the limiting nature of P' in the reaction. In order to show that P' was in fact limiting when the curve levelled off, a mixture of excess A_1 and low P' (prepared as described in Materials and Methods, section 12, Method II part B) was reacted to equilibrium. Additional P' was added and indeed an increase in phenol oxidase produced was seen. Additions of either S or P had no effect. The results of this experiment are summarized in Figure 19.

3. Excess P' and Variable A_1.

In order to determine the effects of variable A_1 concentrations on the final levels of activity reached in the P' and A_1 reaction, experiments were conducted using high constant concentrations of P' and variable concentrations of A_1. A series of incubation mixtures were set up, each containing a high constant concentration of P'. Variable concentrations of A_1 were added to each tube and aliquots were withdrawn at varying times and assayed for phenol oxidase activity. The results of such an experiment are shown in Figure 20. As expected the final level of phenol oxidase produced was dependent on the concentration of A_1 in the activation mixture. In order to show that all the A_1 had been used up in the plateau regions of the curves,

Figure 19. Limiting nature of P' in the reaction with A_1. P' was partially purified over P-60 as described in Materials and Methods, section 12, Method IIB. P, S and A_1 were prepared as described in the text. Each point represents the incubation of 15 µl. of P' with 75 µl. of A_1 in a 125 µl. total volume for the time indicated on the abcissa. At 45 minutes 75 µl. of P' were added to one tube, 25 µl. of S to another and 50 µl. of P to the third.

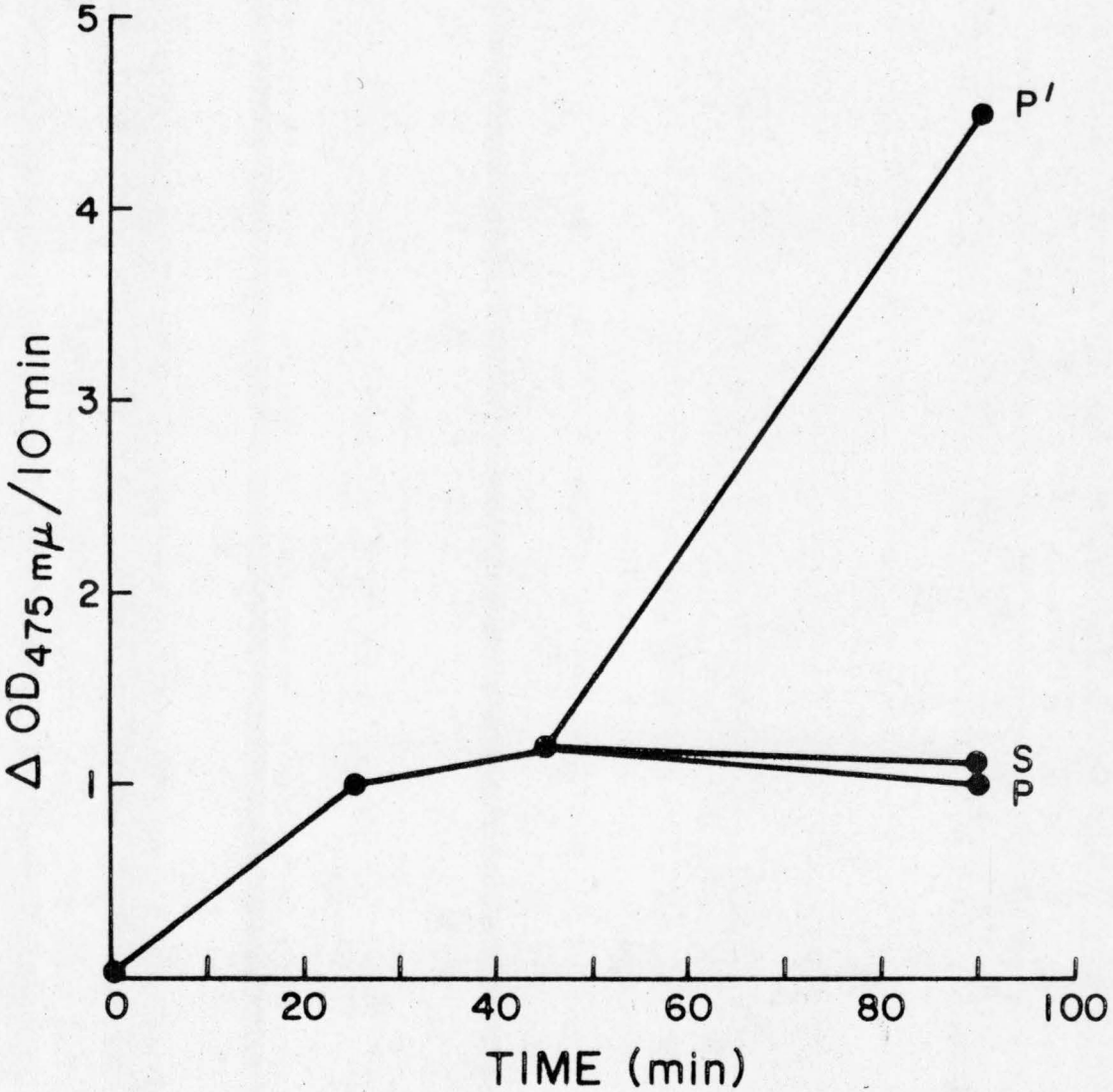
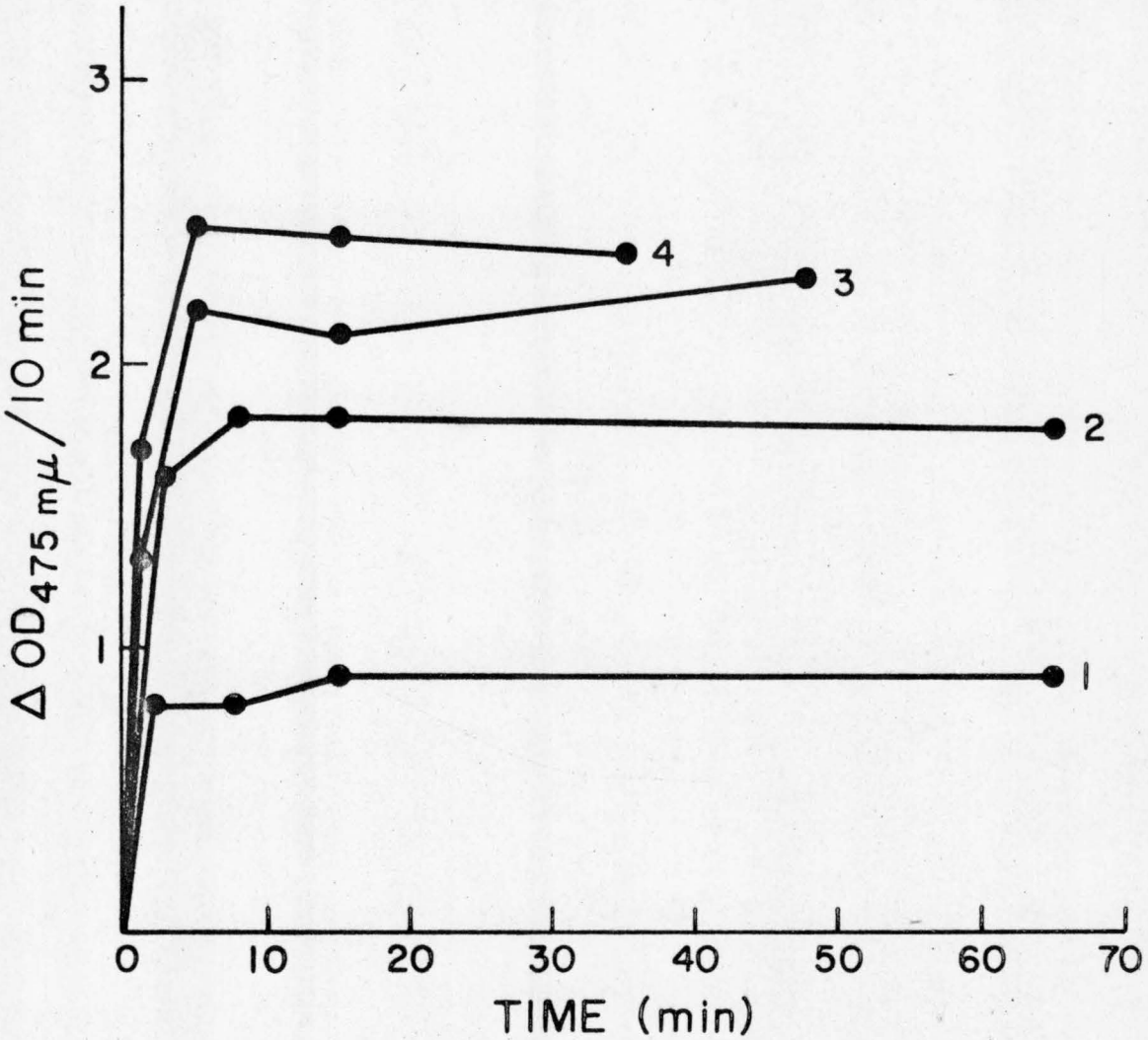

5
4
3
2
1
Δ OD475 mμ/10 min
P'
S
P
0
20
40
60
80
100
TIME (min)

Figure 20. A_1 concentration curves. A_1 and P' were prepared as described in the text.

Curve 1: each point represents 2 µl. of A_1 incubated with 40 µl. of P' in a total volume of 50 µl. for the time indicated on the abcissa followed by a phenol oxidase assay.

Curves 2, 3 and 4 represent the same experiment with 5 µl., 8 µl. and 10 µl. of A_1 per point respectively.


3
2
1
Δ OD475 mμ / 10 min
4
3
2
1
0
10
20
30
40
50
60
70
TIME (min)

additional P' was added to curves 2 and 4 at 60 minutes. This had no effect on the phenol oxidase produced. Additional A_1 added to curves 1 and 2 at 30 minutes also caused no additional phenol oxidase production indicating that no P' was left in these tubes. A large excess of P' was added to tube 3 at 60 minutes followed by addition of excess A_1 30 minutes later. Again no additional phenol oxidase was produced showing that P' activity is unstable in the presence of active phenol oxidase.

4. P' and Pure A_1*.

Highly purified P' (Materials and Methods, section 12, Method II, part D) was reacted with pure A_1, shown to be homogeneous by Meltzer (2). Phenol oxidase was produced in the reaction, strengthening the concept that the P' and A_1 reaction involves only those two components.

Activation of phenol oxidase in crude homogenates results in a highly aggregated product (35). In order to determine if the enzyme produced by partially purified components also aggregates, the following experiment was performed. Active phenol oxidase was produced using P' (Materials and Methods, section 12 Method I) and pure A_1. The reaction mixture was layered on a 5 to 20% (w/v) sucrose gradient and centrifuged at 32,000 g (max.) for 16

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*Prepared by Dr. Paul Meltzer.

hours in the Type SW 39 rotor. All the phenol oxidase activity was found at the bottom of the tube, indicating extensive aggregation. Unreacted A_1 moved about 0.2 cm. into the gradient. A hemoglobin marker moved slightly behind the A_1.

DISCUSSION

1. General.

The existence of 6 protein components, designated Pre S, S, P, A_1, A_2 and A_3, which participate in the activation of phenol oxidase in Drosophila has been described by Mitchell et al. (33, 34). In the work presented here the existence of Pre S, S and P has been confirmed and two additional components, S activator and P', have been described. The partial purification of S, P, P' and S activator has been reported and the interactions of several of the constituents of the activating system have been studied.

2. Preparations of the Components.

A. Purifications.

In interpreting the results presented here it would be useful to know the degree of purity of the components which have been used in the study of the activation process. Most of the components cannot be assayed for in crude homogenates so it is usually not possible to determine the extent of purification in the early stages of a fractionation scheme. Rough estimates however can be made. For instance in the case of the S component, if 100% yield is assumed through the ammonium sulfate fractionation and 25% yield over the hydroxylapatite column, then the S activity would be 1,500 fold purified over the total soluble

protein. In the case of P, 100% yield through the ammonium sulfate step would result in a 70 fold purification through the total scheme. In the case of P', 25% yield through ammonium sulfate fractionation results in 1,000 fold purification through the purification described. These estimates are presented simply in order to give some idea of the likelihood that the various components described could be contaminated with other unknown constituents of the activating system.

B. Resolution.

The S, P and P' components have been shown not to be cross contaminated with each other or with A_1 by tests for enzymatic function. A_2 and A_3 were not assayed for directly but the fractionation data of Meltzer (2) indicate that S, P and P', prepared as described, should not be contaminated with these components.

3. Reactions of the Components.

A series of experiments were conducted in order to determine the nature and sequence of the reactions which are concerned with the production of phenol oxidase. An activation scheme which is consistent with all the data presented is shown in the Introduction, Figure 1. This model depicts the first step in the activation sequence as the reaction between Pre S and S activator to produce S. From the data presented here there is no way of confirming

that the Pre S to S reaction is in fact the first step in the activation of phenol oxidase. The S activator itself could be latent. In this case the apparent lack of S activator in 4 to 8 hour prepupae could be due to the lack of a component required for activation of the S activator. This hypothesis could easily be tested using mixing experiments to see if extracts of other age animals could cause the appearance of S activator in 4 to 8 hour prepupal extracts.

Since the first step in the activation reaction is likely to be the object of regulatory systems it would be of considerable interest to demonstrate the first reaction in the sequence unambiguously. It should be noted that an analysis of the data presented here on the kinetics of activation of S would not be likely to shed any light on this question. The activation curve presented in Figure 11 represents a composite of two reactions. First there is the production of S which will react in the H reaction to produce phenol oxidase and second there is the reaction of S and P, which is also present in the crude Pre S fraction, to produce P' which also reacts in the H assay. As a consequence no conclusions can be drawn from the shape of the curve regarding mechanisms of the S activation.

The second step in the activation reaction as depicted in this model is the reaction of S and P to produce P'. The kinetic data strongly suggest that S acts in a catalytic

fashion on P to produce P'. The possibility that S acts on another component in the P preparation which then reacts with P cannot be excluded until P is more highly purified. The apparently simple kinetics of the S and P reaction seen in Figures 14 and 16 and the fact that the P is approximately 70 fold purified make it appear likely however that the reaction does occur as depicted.

The third step in the activation sequence shown in Figure 1 is the reaction of P' with A_1 to produce phenol oxidase. The reaction of P' with A_1 has been carried out with highly purified components. The A_1 was shown to be homogeneous by Meltzer (2) and the P' has been estimated to be greater than 1,000 fold purified. There is not a strong likelihood that any other components will be shown to participate in this reaction. The precise nature of the reaction however is a matter of considerable conjecture. The end product of the reaction is a highly aggregated molecule. Two types of reaction sequence can be visualized. In the first case P' could act on A_1 to produce an intermediate, either with or without phenol oxidase activity, which then aggregates. In the second case P' could act directly on A_1 to produce an aggregated product without the production of an intermediate. It is not known at this time whether the aggregation reaction is necessary for activation or is a consequence of it. The instability of phenol oxidase to high speed centrifugation (40) suggests the possi-

bility that the aggregated state may be necessary for activity.

Another question of considerable interest concerning this reaction is whether P' is incorporated into the active enzyme. The limiting nature of P' in the reaction with A_1 suggests the possibility that P' does play a structural role in the phenol oxidase molecule. The limiting nature of P' can also be explained by its apparent instability in the presence of active phenol oxidase but this does not preclude the possibility that P' does get incorporated into the active enzyme. One experiment which could be expected to shed some light on this question would be to activate A_1 with P' under conditions which block phenol oxidase activity and see whether or not P' is still limiting in the reaction. A definitive answer to this question will have to await the preparation of homogeneous P'. This could then be labelled and it would be a simple matter to see whether P' gets incorporated into the active product on reaction with A_1.

There is good evidence that the reactions depicted in this model occur and are necessary for the production of phenol oxidase in extracts of Drosophila. The possibility that other reactions occur and are necessary cannot be excluded at this time. In vivo A_2 and A_3 can also be expected to participate in the activation. At present however there are very few data concerning the activation of these com-

ponents. Techniques for the preparation of highly purified A_2 have been worked out (2) and it should be an easy matter to determine if the A_1 activating system also works on A_2. The participation of A_2 and A_3 in the active enzyme is further discussed in Section 5 below.

It is of interest at this time to speculate on the reasons why such a complex activating system is required. This may be related to both the toxicity and function of the enzyme. It is necessary for the animal to maintain unneeded enzyme in an inactive form due to the extreme toxicity of the reaction products of the enzyme. However, the animal must also be able to activate large quantities of phenol oxidase rapidly, within a well defined temporal sequence, during periods of cuticular hardening and pigmentation. The amplifying characteristics of a scheme such as that proposed in Figure 1 allow the production of large quantities of enzyme to be triggered by the release or synthesis of a small amount of S activator. In the absence of this component the animal is protected against possible activation.

4. Mechanisms of the Reactions.

The scheme proposed in Figure 1 does not specify the mechanisms involved in any of the reactions. There is some evidence that at least one of the steps involves limited proteolysis. P has been shown to have a molecular

weight in excess of 60,000 daltons as estimated by gel filtration. P' has been shown to have a molecular weight of approximately 30,000 daltons, again as estimated by gel filtration. If the hypothesis that P' is produced from P is accepted, then a proteolytic mechanism appears likely. Alternatively, the reaction could involve dissociation of subunits, perhaps by reduction of disulphide linkages. Direct verification that the P' activity produced in the S and P reaction is the same as the 30,000 molecular weight P' purified from a 0-35% ammonium sulfate fraction, will involve isolating P' from a purified S and P reaction mixture.

There are no data bearing directly on the mechanism of activation of A_1 by P'. The proenzymes of Bombyx and Calliphora phenol oxidases and A_1 from Drosophila have been activated with proteolytic enzymes (20, 25, 2). In all three cases however, only a small fraction of the potential activity was released. The suggestion has been made that the natural activation may also involve limited proteolysis (20, 25). The activation by proteolytic enzymes has no direct bearing on the question of natural activation but the proteolytic activation mechanism is an attractive hypothesis. It should be possible to test this mechanism in the A_1 and P' reaction by stopping the activation reaction with strong denaturing agents and searching for intermediates with an altered molecular weight. If the

aggregation reaction were to occur rapidly and produce a non-dissociable product, it could prove difficult to isolate the putative intermediate.

As mentioned above, the question of the participation of P' in the aggregation reaction must also be considered in a discussion of the mechanism of activation of A_1. An alternative hypothesis to the proteolytic mechanism is that A_1 depends on the aggregated state for activity and P' is required for formation of the aggregate but does not structurally alter the A_1 molecule. The role of P' in the formation of the aggregate could be as a structural component necessary for the stability of the aggregate. P' acting in this way might also catalyze the formation of covalent bonds in the aggregate. P' could also function in the formation of an aggregate by catalyzing the formation of covalent bonds between A_1 molecules without functioning in a structural role.

5. Further Experiments.

Further studies on the properties of the aggregated enzyme should be possible using purified P' and A_1. Blumenthal's work (48) indicates that the enzyme produced by activation in crude homogenates is at least partially covalently cross linked. The dissociability of the aggregate formed in the purified system could be determined to see if covalent cross linking also plays a role

in this system. If covalent cross linking does occur in the purified system it should be possible to exclude rigorously small molecules from the system to determine if small molecules are required for cross linking. If A_2 and A_3 were prepared in purified form it would be possible to determine the effects of variations in the proportions of A components on the degree of aggregation, substrate specificity and density of the enzyme produced.

In light of Lewis and Lewis's demonstration of the participation of lipid in the activation reaction (32) and in view of Blumenthal's observation that banded phenol oxidase contains lipid (48) it would be of interest to determine the effect of exogenous lipid on the activation and aggregation in a purified system.

Another problem which should be amenable to analysis with purified components concerns the function of the phenol oxidase system. Phenol oxidase has been proposed as a major structural protein in the formation of cuticle as well as being involved in tanning and hardening of the integument. The cuticular function of phenol oxidase has however not been rigorously proven. It has been suggested by Meltzer (2) that a definitive answer to this question could be arrived at by preparing labelled A_1, injecting it into the hemolymph and observing if radioactivity gets incorporated into the cuticle. As he points out this would depend on the assumption that radioactive A_1

injected into the hemolymph could reach the site of cuticle synthesis. Another possibility would be to prepare labelled antibodies directed at A_1 in an attempt to localize A_1 in the cuticle using techniques of immunofluorescence. This would depend on the assumption that the antigenicity of A_1 is not too greatly altered during incorporation into the cuticle. The experiment might also be done by preparing antibodies to phenol oxidase prepared from purified components on the assumption that such material might more closely resemble the material in the integument than A_1 does.

With a detailed knowledge of the activation reaction it should be possible to bring genetic analysis to bear on the phenol oxidase system. Biochemical analysis of known body color mutants and strains with cuticular abnormalities might be expected to provide information concerning the genes responsible for the synthesis and regulation of phenol oxidase components. As an interesting example of this type of analysis, Mitchell (35) has shown that in the period from 70 hours following puparium formation to emergence, extracts of straw mutant flies activate considerably more slowly than extracts of wild type flies although the potential maximum activity is higher in the mutants. This slow period corresponds to the time in which pigmentation normally occurs in wild type flies. It would be of interest to compare the activating system of straw flies with that of wild type to see if it were possible to localize

the difference to a single component of the activating system. Mitchell has also investigated the density distribution of phenol oxidases from several body color mutants and found that density distributions were specific to a mutant and developmental stage (40). Further biochemical analysis of these strains would also be of interest. Another strain of potential interest for this type of analysis is the alpha strain isolated by Lewis and Lewis (32). They suggest that the alpha gene affects the primary structure of the enzyme since phenol oxidase isolated from this strain is more thermolabile than the wild type enzyme.

6. Possible Regulatory Mechanisms.

Through the analysis of genetic mosaics it has been shown that pigmentation of a given portion of the cuticle is regulated by the underlying epidermal cells (49). A number of mechanisms can be proposed to explain the regulation of phenol oxidase activity by these cells. Two main levels of regulation can be conceived of, at the substrate level, and at the enzyme level.

Concerning regulation at the substrate level, Mitchell (50) has pointed out that all degrees of hardness and pigmentation observed in cuticular structures could be regulated by the amount and type of substrate presented to the enzyme. The epidermal cells could be expected to play

an important role in the regulation of substrates available to the enzyme by controlling the pathways of substrate synthesis. It will be necessary to have a greater understanding of the pathways of phenolic metabolism before it will be possible to understand fully the potential control systems operating at the substrate level.

At the enzyme level the epidermal cells can be expected to regulate cuticular hardening and darkening through the control of the amount and type of components released into the cuticle. Substrate specificity and the structural properties of the enzyme could be regulated by controlling the relative amounts of A components secreted into the cuticle. Variations in the amount of activating components can be expected to affect enzyme levels and possibly also the structural properties of the enzyme produced. In this regard it is likely that the first component in the activation scheme is subject to tight regulation. The properties and tissue localization of this component, possibly the S activator, merit further studies and must be described before the in vivo process of activation can be understood.

In summary, it appears likely that genetic and biochemical analysis of phenol oxidase will provide useful information concerning the regulation of cuticle biosynthesis.

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PART II

STUDIES ON SERUM INSULIN IN NORMAL SUBJECTS AND DIABETICS

INTRODUCTION

1. General.

The relationship of pancreatic hypofunction and diabetes mellitus was first described by von Mering and Minkowski (1) in 1889. It was not until 1922 however that Banting and Best were able to isolate the active pancreatic principle (2). The isolation of crystalline insulin was accomplished by Abel in 1926, this being the first isolation of a crystalline protein with specific biological properties (3). In spite of the fact that insulin and its relationship to diabetes have been known for so long, several problems remain concerning the nature and function of the hormone and its connection with diabetes mellitus. In particular, the nature of the circulating hormone and the primary metabolic effects associated with insulin action are poorly understood. In addition, little is known concerning the primary molecular defects associated with diabetes. Although diabetes can be described as a functional lack of insulin activity, the reasons for this lack are not understood.

This thesis is concerned with an electrophoretic analysis of serum insulin, which was carried out to elucidate further the nature of the circulating hormone and to test the hypothesis that at least some forms of diabetes

may be the result of the secretion of polymorphic forms of insulin with altered biological properties. To provide a background for the work presented here the remainder of this introduction is devoted to a brief review of insulin biosynthesis, the nature of serum insulin and possible molecular mechanisms associated with the onset of diabetes. No mention is made of the metabolic effects of insulin or of the metabolic defects associated with diabetes. These subjects have been extensively reviewed elsewhere (4, 5, 6).

2. Biosynthesis of Insulin.

The insulin molecule consists of two chains, the A and the B chains, of 21 and 30 amino acids respectively, which are joined by two disulphide linkages (7). Following this discovery considerable speculation arose concerning the mechanism of its biosynthesis. The two possibilities most actively considered were combination of separately synthesized chains (8) and biosynthesis from a single polypeptide precursor (9). The validity of the second hypothesis was shown by Steiner and co-workers (10, 11, 12, 13). While studying the incorporation of labelled amino acids by an islet cell adenoma prepared from a human pancreas, a 10,000 molecular weight peptide was isolated and shown to exhibit a precursor product relationship with insulin. The peptide was found to be immunologically related to insulin and was shown to be converted into a molecule differing from insulin

by only one amino acid on incubation with trypsin. Further studies on isolated islet cells from rat pancreas confirmed these findings. The insulin precursor was named proinsulin and was shown to be a single polypeptide chain consisting of the A and B chains of insulin linked by a connecting peptide. Amino acid sequence data and immunological analysis of proinsulins from several species indicate considerable heterogeneity in the connecting peptide (14, 15, 16). These results suggest that the connecting segment may have few specific structural requirements. The mechanism of conversion of proinsulin to insulin has not been worked out but an intermediate form of the molecule has been found which was shown to consist of proinsulin in which cleavage of a peptide bond had occurred to produce an A chain connected by disulphide linkages to the rest of the peptide.

3. Nature of Serum Insulin.

A. General.

Insulin has never been extracted from blood in quantities sufficient to permit chemical analysis. As a result the term serum insulin refers only to an activity in serum which mimics that of crystalline insulin in one or more of a variety of systems. A prime difficulty in this field is determining whether the activity being studied is in fact due to pancreatic insulin.

B. Assays.

Two assays based on the biological activity of insulin and one based on its immunological reactivity are in common usage for the study of serum insulin. The bio-assays involve either measurement of glucose uptake by the isolated rat hemidiaphragm or measurement of the conversion of glucose to CO_2 by the rat epididymal fat pad. The radio-immunoassay involves a competition reaction between labelled and cold insulin for binding sites on an anti-insulin antibody. It is described in more detail in Results, section 1.

The fat pad assay almost certainly responds to substances in serum which are unrelated to pancreatic insulin. Only a small quantity of the insulin-like activity measured in this assay is suppressed by anti-insulin antibodies and the activity persists in the serum of pancreatized animals (17). The activity measured by the hemidiaphragm method, on the other hand, is suppressed by anti-insulin antibodies and is absent in the serum of pancreatized animals (17). There is however a serious lack of proportionality on dilution. The radio-immunoassay measures immunoreactive insulin and is independent of the biological action of the hormone. The validity of this assay has been evaluated (18, 32) and the results have been favorable. It appears likely that the immunoassay does not respond to substances in serum unrelated to pancreatic insulin. The question of whether

the assay measures the true biological activity of serum insulin is more open to debate. Data relevant to this question are discussed in parts C and D below.

C. Circulating Proinsulin.

Steiner et al. (19) found that proinsulin was present in the serum of every subject they measured. Proinsulin reacts in the immunoassay and was found to account at times for up to 50% of the immunoassayable insulin. Proinsulin has low biological activity (16) so this could cause immunoassayable insulin levels to be falsely elevated over the true biological activity. The physiological significance of the presence of proinsulin in serum is not known.

D. Bound Insulin.

Several investigators, using the hemidiaphragm assay, have provided evidence that some of the serum insulin is associated with a high molecular weight fraction of the serum proteins (20, 21, 22). Workers using the immunoassay method have been unable to confirm this observation (17, 23). This discrepancy can be explained in one of two ways. The high molecular weight insulin-like activity could represent true biologically active insulin bound to a carrier protein and not susceptible to immunological assay or an artefactual activity similar to that seen in the fat pad assay.

Disruption of the putative insulin-carrier complex in an attempt to release immunoreactive insulin might be expected to resolve this controversy.

E. Half-life of Serum Insulin.

Several investigators have measured the half-life of insulin in human serum. Using immunoassay techniques, the value for both endogenous and exogenous insulin has been estimated at between 9 and 16 minutes for both non-diabetics and diabetics who have never been treated with insulin (24, 25, 26, 27). The half-life of the hormone in insulin treated diabetics has been estimated to be on the order of several hours (28, 29). The longer half-life is reported to be due to the binding of insulin by antibodies present in the serum of these individuals.

4. Possible Molecular Defects in Diabetes.

A. General.

A knowledge of the precise molecular defects involved in the onset of diabetes would be potentially useful in devising methods for the diagnosis and treatment of the disease. It would also be useful in an analysis of the inheritance of the disorder. Diabetes has long been known to have a genetic component but the exact mode of inheritance is unknown. Part of the problem is undoubtably

due to difficulties involved in recognizing the phenotype. Diabetes is diagnosed through observation of disorders in carbohydrate metabolism but the observed disorders could arise as the result of a number of different primary molecular mechanisms. If this were the case, meaningful genetic analysis would have to await the elucidation of these mechanisms.

The functional lack of insulin activity in diabetes can be explained by one of three general mechanisms, which are not mutually exclusive. First, normal insulin could be synthesized and its action could either be blocked by an antagonist or the tissues might for some reason be unable to respond. Second, abnormal or polymorphic forms of insulin with defective biological properties could be synthesized and third, defects in the biosynthetic or secretory machinery could block the production or release of insulin. There are few reliable data concerning the first possibility. The second and third hypotheses are discussed below in parts B and C.

B. Polymorphic Forms of Insulin.

Insulin polymorphism has been observed only in the rat which produces two forms of the hormone differing in amino acid sequence (30). Each individual appears to produce both types of molecule. Considerable heterogeneity is observed when insulin from most species, including humans,

is analyzed by disc electrophoresis (31). This is not the result of genetic polymorphism however, but appears rather to be the result of progressive deamidation of the molecule, perhaps during isolation and purification.

The possibility that an abnormal or polymorphic form of insulin might account for the symptoms of diabetes was actively considered following Berson and Yalow's demonstration that high levels of immunoreactive insulin are present in the blood of diabetics (32). They suggested that their results could be explained by assuming that an abnormal insulin with defective biological activity but near normal immunological reactivity was present. This hypothesis has been extensively investigated by Roy and co-workers (33, 34, 35). They demonstrated that immunoassayable insulin in the serum of juvenile diabetics is more resistant to the action of insulinase than insulin from the serum of normal subjects. They were also able to show that immunoassayable insulin isolated from diabetic pancreases is less effective in promoting glucose uptake by the rat hemidiaphragm than is insulin from normal pancreases. Roy suggests that these results support the notion that an abnormal insulin, perhaps resulting from gene mutation, is present in diabetics. These results, and also those of Berson and Yalow can also be explained by assuming that the abnormal insulin is in fact proinsulin. Proinsulin reacts in the immunoassay (19) and has little biological activity (16). Recently Roy *et al*.

(36) have demonstrated that insulin, isolated from diabetic pancreases and shown to be free of proinsulin, is less effective than normal insulin in stimulating uridine uptake by human fibroblast cultures. It is not known, however, how specific this assay is for insulin activity.

Kimmel and Pollock (37) have reported a possible amino acid substitution in insulin isolated from a diabetic pancreas but the amounts analyzed were too low for reliability.

Brunfeldt et al. (38) were unable to find any chemical or biological differences between insulin isolated from 19 pooled diabetic pancreases and normal human insulin. This does not rule out the possibility that a portion of the diabetic insulin was abnormal however.

It would appear that the question of the existence of polymorphic forms of insulin in diabetics is still an unsettled problem.

C. Defects in Biosynthesis and Secretion.

Steiner (16) has suggested that defects in insulin biosynthesis could account for at least some types of diabetes. Mutations affecting the structure of proinsulin could block its conversion to insulin. Similarly, mutations in the enzymes responsible for the conversion of proinsulin to insulin could also block the synthesis of the hormone. These hypotheses should be amenable to experimentation but there are few data available at this time.

Impaired secretion of insulin has also been advanced as a possible cause of diabetes (39). Again, there are few reliable data concerning this possibility. However, the fact that some diabetics can be treated with sulfonylurea type drugs, which act by stimulating the release of insulin from the pancreas (40), suggests that impaired secretion may be a factor in at least some forms of the disease.

5. Research Outline.

The work presented here is concerned with further investigations of the possibility that abnormal or polymorphic forms of insulin might be present in the serum of diabetics. Disc electrophoresis on polyacrylamide gels was used for the analysis of normal and diabetic serum insulin. This involved the development of techniques for the partial purification of serum insulin and for its detection on polyacrylamide gels.

Materials and Methods

1. Insulin Assay.

Insulin was assayed by Method C of the radio-immunoassay developed by Hales and Randle (41).

2. Preparation of Serum.

Blood (10-15 ml.) was collected* and allowed to clot. Following clot formation, the sample was centrifuged for 20 minutes at 10,000 r.p.m. in the SS-34 rotor of the Servall centrifuge. Aliquots (2 ml.) of the supernatant serum were stored frozen at -20°C.

3. Preparation of Serum Insulin.

A. Bio-Gel P-30 Chromatography.

Bio-Gel P-30 (100-200 mesh) was prepared exactly as recommended by the manufacturer (42) and a 3.2 x 80 cm. column was poured. Hemoglobin (10 mg.) was dissolved in 4 ml. of serum and the sample was applied to the column and eluted with 0.05 M $(NH_4)_2CO_3$ pH 9.0 at a flow rate of 28 ml. per hour. Fractions (4.5 ml.) were collected. Material eluting in the void volume, as determined by the elution of the hemoglobin marker, was discarded and subsequent fractions,

*Courtesy of Dr. S. Gordon Ross.

corresponding to 3 void volumes, were pooled.

B. Ultrafiltration.

In some experiments the pooled fractions were desalted using ultrafiltration in an Amicon apparatus. A UM-2 membrane was used.

C. Lyophilization.

The pooled fractions were lyophilized to dryness. The dried material was dissolved in 200 µl. of 0.01 M phosphate pH 6.7 for use in electrophoresis experiments.

4. Disc Electrophoresis.

Disc electrophoresis on polyacrylamide gels was carried out by the method of Davis (43). The concentration of acrylamide was 20% and the concentration of methylenebis-acrylamide was 0.15%. Electrophoresis was carried out at 2 ma per tube constant current for 30 minutes following the time the tracking dye reached the end of the gel. Electrophoresis was done at 0-4°C.

5. Gel Fractionation.

Polyacrylamide gels were fractionated by the method of Ward (44). This technique involves controlled extrusion of the gel through a small orifice. Usually, 75 fractions were obtained from a 6 cm. gel. Each fraction was incubated

at 0°C. in 250 µl. of 0.01 M phosphate pH 6.7 for 24 hours following which 100 µl. aliquots were withdrawn for the assay of insulin.

6. Measurement of Radioactivity.

Radioactivity was determined in a Nuclear Chicago model 7-24 liquid scintillation counter. The scintillation fluid consisted of ethanol (150 ml.), dioxane (300 ml.), napthalene (50 g.), 2,5-diphenyloxazole (5 g.) and toluene to 1 liter. In general, 10,000 counts were obtained for each sample.

7. Reagents.

All reagents were reagent grade. Antibodies and iodinated insulin for use in the radio-immunoassay were obtained from Amersham-Searle corporation. Hemoglobin, bovine crystalline insulin, and bovine albumin powder were obtained from the Sigma Chemical Company.

RESULTS

Section 1. Assay.

A. General.

The assay of insulin in concentrations comparable to those found in human serum requires very sensitive techniques. The two main types of assay which are suitable for this purpose are bioassays and radio-immunoassays. Bioassays generally involve the measurement of a biological effect of insulin on isolated tissue. Radio-immunoassay techniques depend solely on the immunological reactivity of the insulin. A radio-immunoassay was used in the experiments reported here since a primary objective of these experiments was the detection, in diabetic serum, of altered insulin molecules with defective biological properties. These would not be expected to react in a bioassay but it was hoped that immunological cross reactivity would remain.

The radio-immunoassay depends on a competition reaction between a constant concentration of labelled insulin and variable concentrations of the unknown for binding sites on anti-insulin antibodies. The amount of radioactivity bound is inversely proportional to the insulin concentration of the unknown. Different assays vary in the techniques

used to separate the bound and unbound insulin in the incubation mixture. In the assay of Hales and Randle (41) which was used in these experiments, the separation is obtained by membrane filtration. The insulin-antibody complex is retained while the unbound insulin passes through. The filter discs can then be counted directly to determine the amount of labelled insulin bound in the competition reaction.

B. Standard Curve.

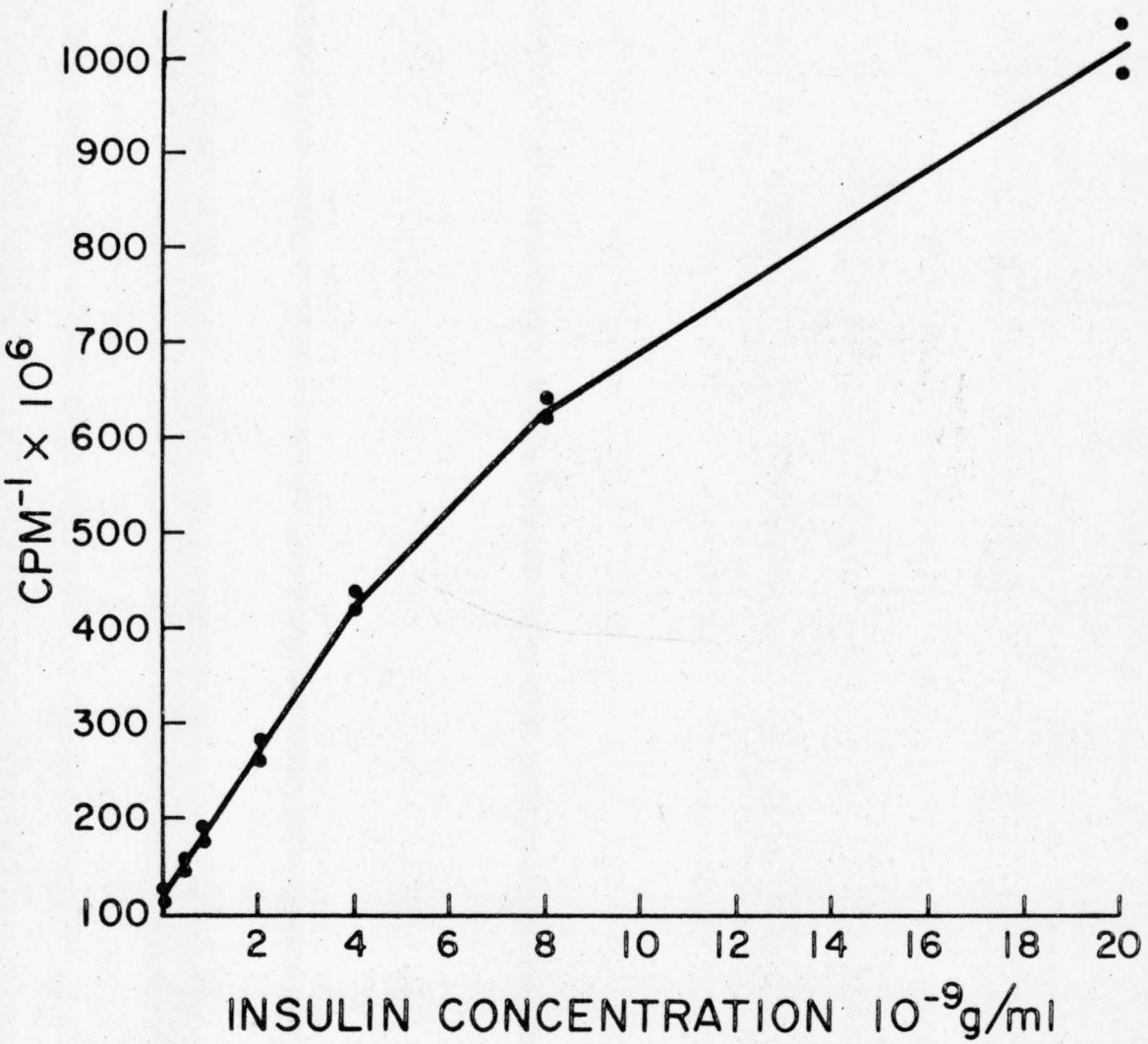
A standard curve for the Hales and Randle assay is shown in Figure 1. The reciprocal of the bound radioactivity is plotted against insulin concentration. The assay is linear at low concentrations and is sensitive to changes in insulin levels over a 40 fold range. The linear portion of the assay corresponds to insulin concentrations normally found in human serum.

The assay was to be used for the determination of insulin eluted from polyacrylamide gels. Experiments were conducted to determine if polyacrylamide gel fractions or exogenous proteins had any effect on the standard curve. No effects were observed.

Section 2. Preparation of Insulin From Human Serum.

Due to the low concentrations of insulin found in serum it was not possible to electrophorese serum samples

Figure 1. Standard curve for the insulin assay. Bovine crystalline insulin was used as a standard. Dilutions were prepared and assayed as described in Materials and Methods.


1000
900
800
700
600
500
400
300
200
100
CPM^{-1} X 10^{6}
2
4
6
8
10
12
14
16
18
20
INSULIN CONCENTRATION 10^{-9}g/ml

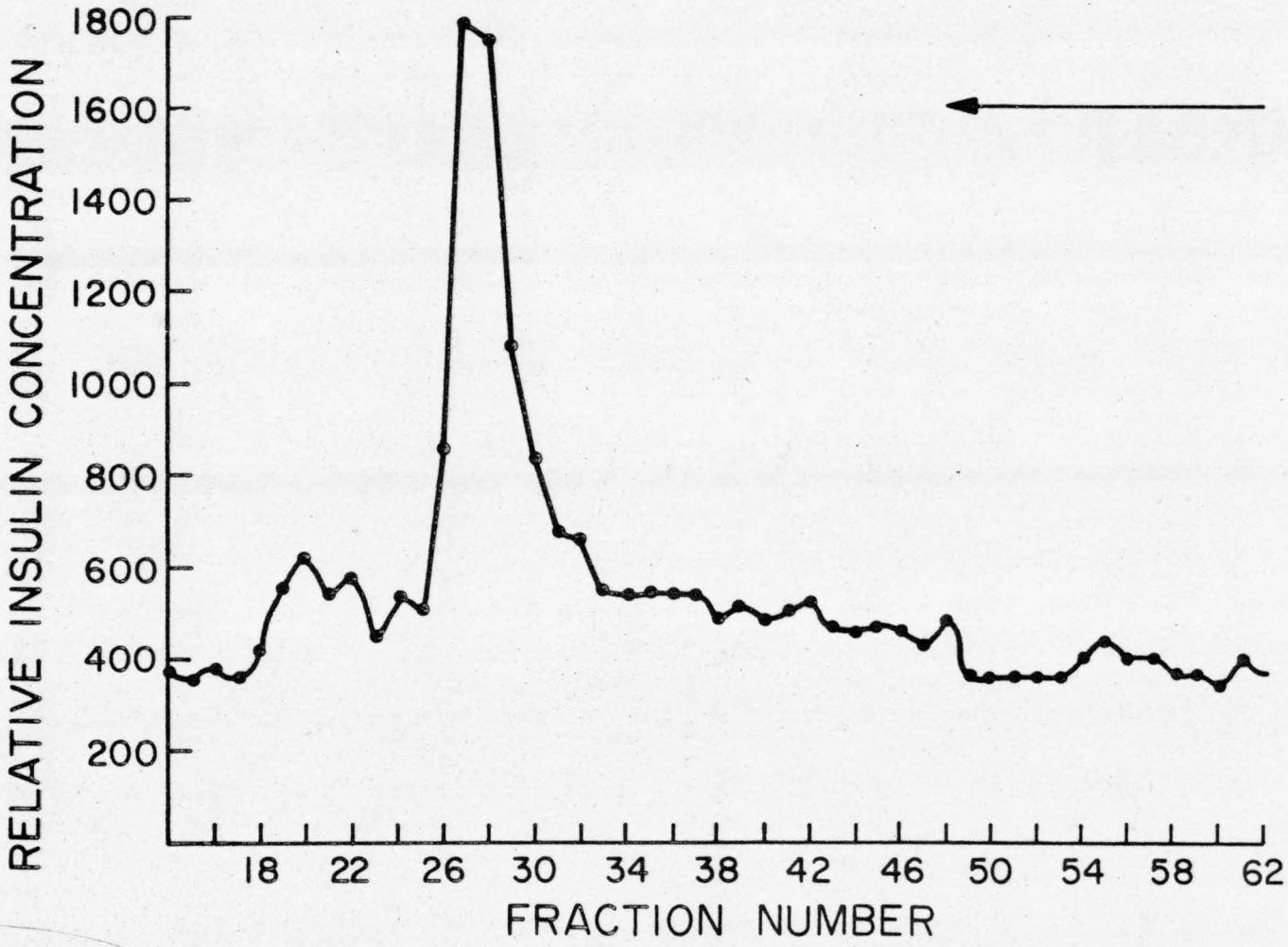
directly. A partial purification and concentration of the insulin activity was required. Because of the low molecular weight of insulin as compared to the bulk of serum proteins, this was easily accomplished by gel filtration on Bio-Gel P-30 as described in Materials and Methods, section 3. In order to allow concentration of the insulin-containing fractions by lyophilization, the column was eluted with a volatile buffer, 0.05 M $(NH_4)_2CO_3$. In this way it was found possible to prepare the insulin from 4 ml. of serum in a volume of 200 µl. with protein concentrations low enough to allow electrophoresis of the sample.

Section 3. Electrophoretic Analysis of Serum Insulin.

A. Normal Human Serum.

Experiments involving electrophoresis of insulin from normal serum were conducted in an attempt to determine the normal electrophoretic pattern of serum insulin. Insulin, prepared from 2 ml. of human serum (Materials and Methods, section 3), was electrophoresed on a 20% acrylamide gel and the gel was fractionated as described in Materials and Methods, section 5. Aliquots of the fractions were assayed for insulin activity (Materials and Methods, section 1). The results of a typical experiment are shown in Figure 2. A major peak of immunoreactive insulin was observed, preceded by a cluster of more rapidly moving

Figure 2. Electrophoresis of normal insulin. An aliquot (100 µl.) of normal insulin (Materials and Methods, section 3) was electrophoresed on a 6 cm. 20% acrylamide gel. Fractions (75) were collected and assayed for insulin activity. Relative insulin concentration refers to the reciprocal of the counts per minute obtained in the assay times 10^6. The direction of migration is indicated by the arrow.

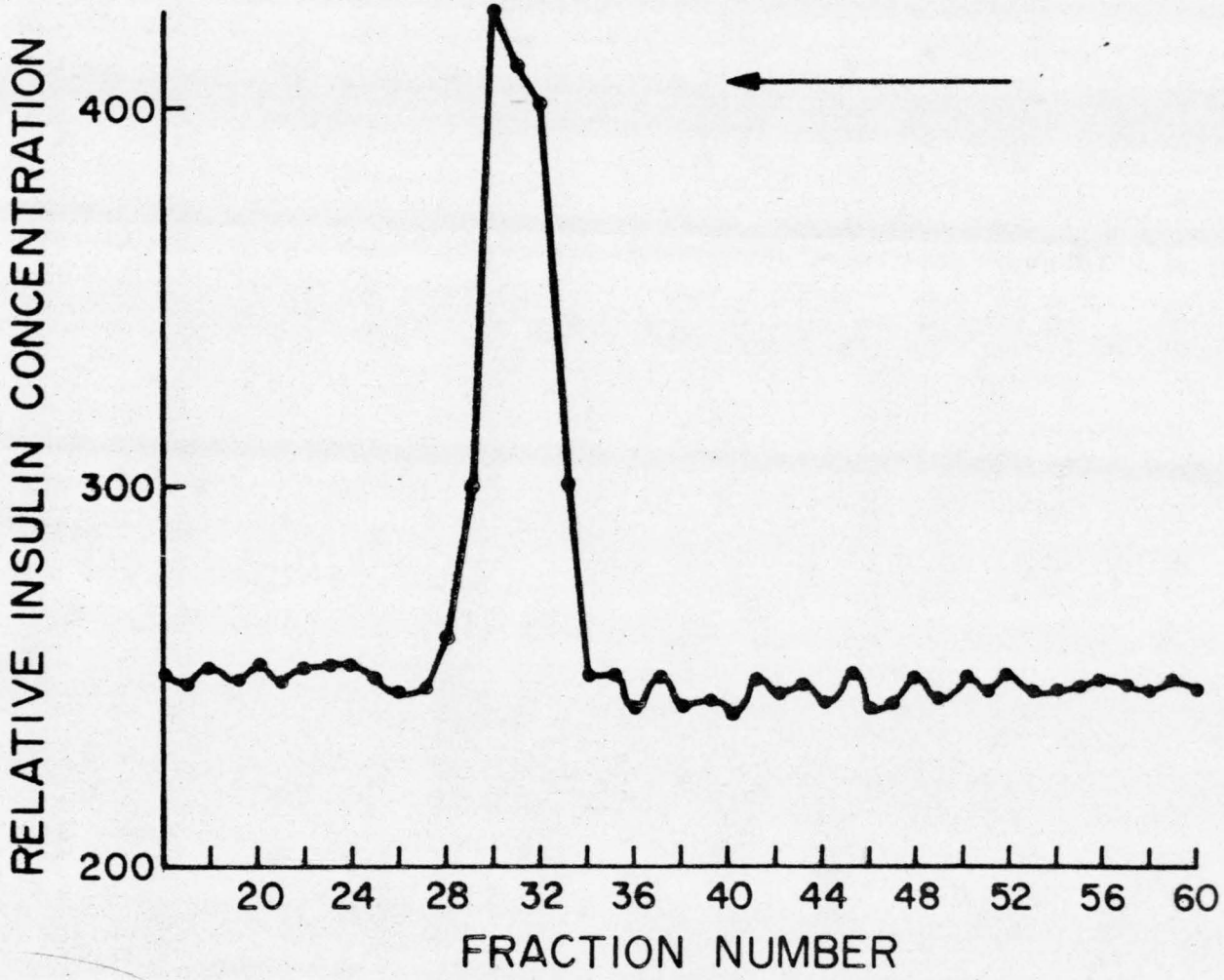

RELATIVE INSULIN CONCENTRATION
1800
1600
1400
1200
1000
800
600
400
200
18
22
26
30
34
38
42
46
50
54
58
62
FRACTION NUMBER

minor peaks. The faster moving peaks were always seen to be present in repeat experiments and were sometimes resolved into two or three components. An attempt was made to determine if the faster moving components were being generated from the major peak under the conditions of the experiment. An aliquot from the peak tube of activity in an experiment such as that depicted in Figure 2 was incubated for 24 hours in 0.05 M $(NH_4)_2CO_3$ and re-electrophoresed on a 20% acrylamide gel. Fractions were taken and assayed for insulin activity. The results of this experiment are depicted in Figure 3. In this case no evidence of faster moving peaks was observed. It is possible that a small amount of the faster moving material could have been generated during this procedure and not have been detected in the assay but it doesn't appear that significant conversion occurred.

B. Diabetic Serum.

Experiments were conducted in an attempt to determine if electrophoretic abnormalities could be detected in the insulin isolated from the serum of diabetics. Insulin was prepared from the serum of 12 diabetic patients, 5 of whom were receiving insulin therapy. In agreement with the results of Berson and Yalow (32), serum levels of the hormone in all of the diabetics were comparable to or higher than those found in normal subjects. Insulin from each of

Figure 3. Re-electrophoresis of normal insulin. An aliquot (100 µl.), obtained from the peak tube of an experiment such as that described in Figure 2, was electrophoresed on a 6 cm., 20% acrylamide gel. Fractions (75) were collected and assayed for insulin activity. The direction of migration is indicated by the arrow.


RELATIVE INSULIN CONCENTRATION
400
300
200
20
24
28
32
36
40
44
48
52
56
60
FRACTION NUMBER

the diabetics was mixed with normal human insulin and electrophoresed. Gels were fractionated and assayed for insulin activity as described in Materials and Methods. In the case of 10 of the diabetics, no differences could be detected between the electrophoretic pattern of the mixture and that of the normal insulin alone. In the case of two diabetics, designated GA and GWII, who had been receiving insulin therapy, the electrophoretic pattern of the mixture differed markedly from that observed with normal insulin. In both cases, the pattern observed was very similar. The results of a mixing experiment with insulin isolated from GA are presented in Figure 4. This pattern was observed in seven repeat experiments. In no case was a normal pattern observed with mixtures of insulin from GA and a normal subject. These results could be caused by the mixing of two samples with slightly different electrophoretic mobilities. The A and B peaks could be the two main insulin peaks. The C peak would then be part of the fast moving material associated with peak A. An electrophoretic analysis of the insulin isolated from GA alone is shown in Figure 5. This pattern does not seem to differ significantly from that observed with normal insulin. A peak similar in position to the small peak A observed trailing the main fraction was occasionally observed in analyses of normal insulin.

Several attempts were made to obtain a better resolution

Figure 4. Electrophoresis of mixed diabetic and normal insulin. Aliquots (100 µl.) of insulin prepared from normal serum and diabetic serum (GA) were mixed and electrophoresed on a 6 cm., 20% acrylamide gel. Fractions (75) were collected and assayed for insulin activity. The direction of migration is indicated by the arrow.

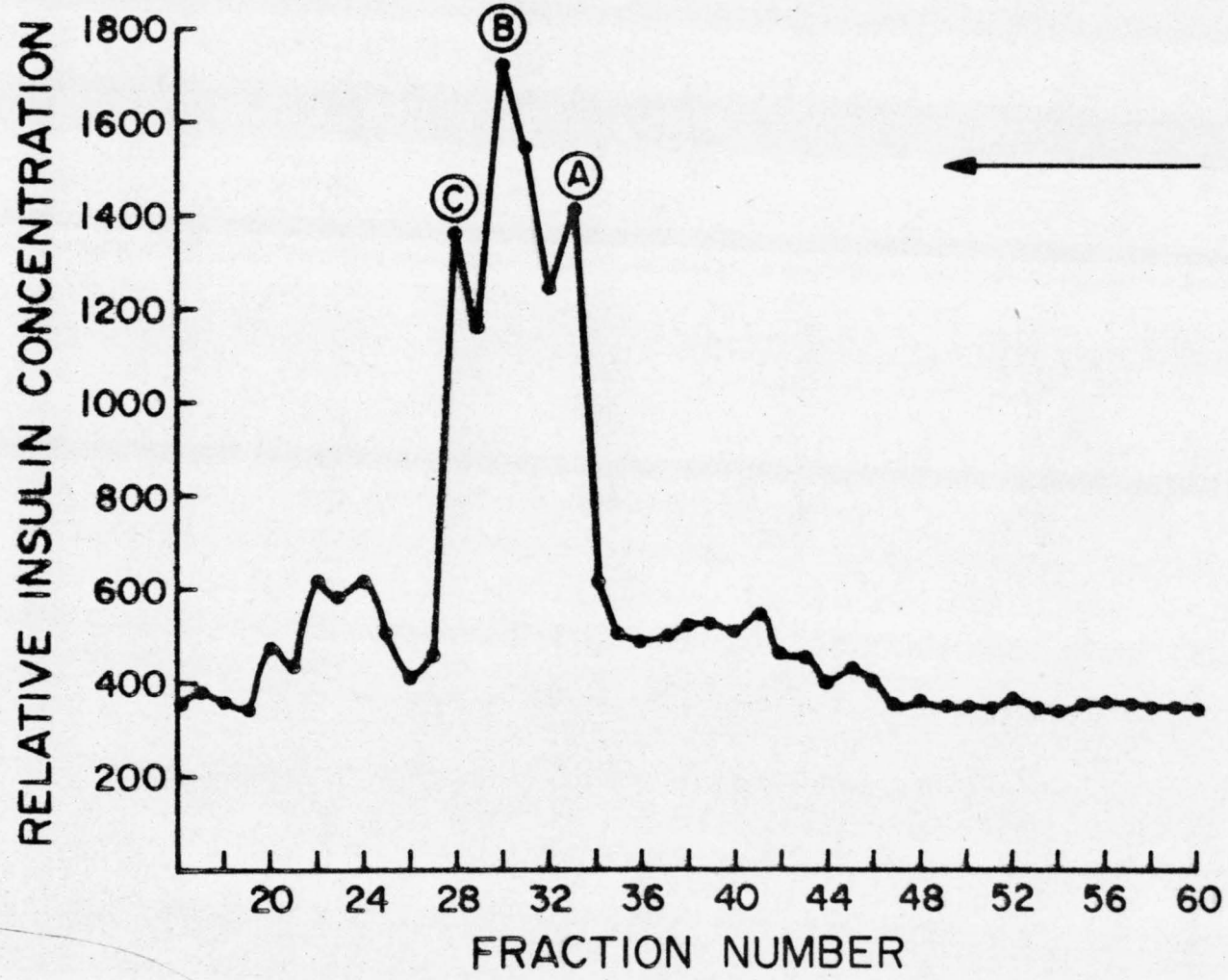
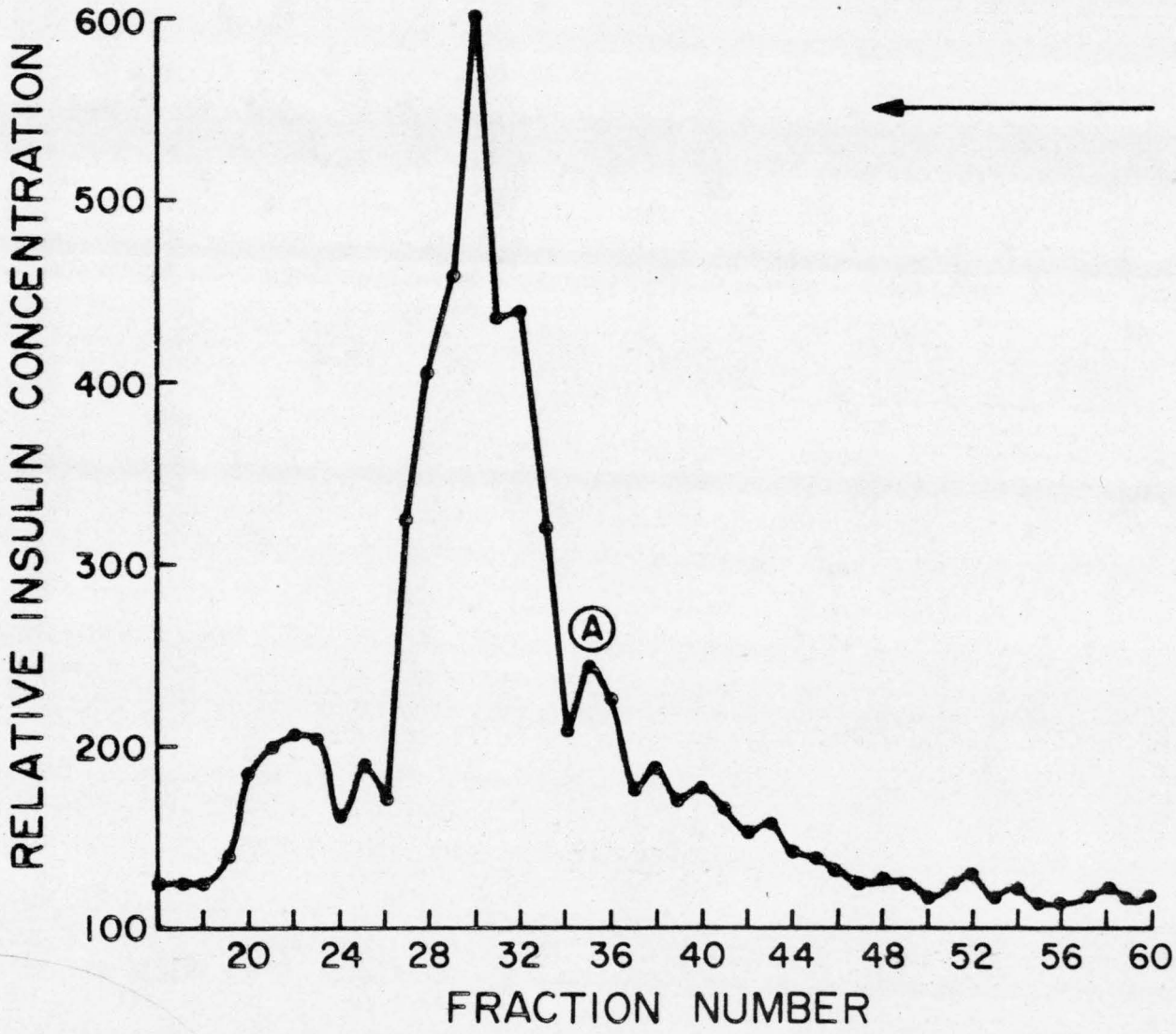

RELATIVE INSULIN CONCENTRATION
1800
1600
1400
1200
1000
800
600
400
200
C
B
A
20
24
28
32
36
40
44
48
52
56
60
FRACTION NUMBER

Figure 5. Electrophoresis of diabetic insulin. An aliquot (100 µl.) of insulin prepared from the serum of the diabetic, GA, was electrophoresed on a 6 cm., 20% acrylamide gel. Fractions (75) were taken and assayed for insulin activity.


RELATIVE INSULIN CONCENTRATION
600
500
400
300
200
100
A
20
24
28
32
36
40
44
48
52
56
60
FRACTION NUMBER

of the main peaks of normal insulin and that isolated from GA. These included isolation of insulin from peak tubes of separately run GA and normal insulin followed by mixing and re-electrophoresis. Difficulties in handling the very low concentrations of insulin involved precluded the obtaining of reproducible results. An attempt was made to cast 12 cm. gels of 20% polyacrylamide in the hope that the increased length would allow better resolution. It was found that gels over 6 or 7 cm. invariably broke when polymerization occurred. Alterations in the temperature of polymerization or concentrations of TEMED, BIS, and ammonium persulfate failed to solve the problem. Attempts to cast the gel in sections were also unsuccessful. Gels formed from 10% acrylamide could be cast in long tubes but failed to provide the necessary resolution.

DISCUSSION

1. General.

It has proved possible to measure the electrophoretic mobility of serum insulin accurately on analytical polyacrylamide gels. The electrophoretic patterns of normal and diabetic insulin have been analyzed and an indication that differances may occur has been noted.

2. Electrophoretic Pattern of Normal Insulin.

The electrophoretic pattern of normal insulin was found to be very similar to that observed on electrophoresis of human crystalline insulin (45). When crystalline human insulin is electrophoresed, a single major protein band is observed, preceded by two less intensely staining minor bands. One or two very faint bands are also observed trailing the major band. It has been suggested that the minor bands represent desamido forms of insulin produced during the purification procedure (31). The evidence presented here suggests that these minor forms of insulin are also present in the circulation. The potential physiological significance of this observation is not known.

3. Electrophoretic Patterns of Diabetic Insulin.

Insulin from the serum of diabetics was analyzed in an

attempt to locate electrophoretic variants. The data presented in Figure 4 suggest a difference between the electrophoretic mobilities of normal and diabetic insulin. The patterns of normal insulin and that from the diabetic GA are very similar but on mixing a complex pattern is observed suggesting a difference in electrophoretic mobilities. A major objection to this observation is the lack of resolution involved. The slow moving peak A in Figure 4 is resolved by only one point. Further resolution will be necessary before the existence of an electrophoretic variant in this serum can be confirmed. As indicated, efforts in this regard have been unsuccessful so far. A more serious objection concerns the fact that the subject was receiving insulin therapy and the possibility that the altered form of insulin represents exogenously administered hormone must be considered. Evidence that this is not the case comes from analysis of diabetics who had received comparable amounts of the same type of insulin and whose electrophoretic patterns were indistinguishable from normal insulin. Unambiguous demonstration that an electrophoretic variant exists will have to come from individuals who have not received insulin therapy.

If it is possible to demonstrate that such a variant exists, then genetic analysis using the altered form as a phenotypic marker would be expected to provide useful information concerning the mode of inheritance of the disease.

Such information could be of great value in genetic counselling and related fields.

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List of Abbreviations

(Parts I and II)

BIS ..... N,N'-methylenebisacrylamide

TEMED ..... N,N,N',N'-tetramethylethylenediamine

OD ..... optical density

CPM ..... counts per minute