PEPTIDYL ACYLATING AGENTS IN THE PUPAE OF *DROSOPHILA MELANOGASTER* AND THEIR POSSIBLE RELATIONSHIP TO PROTEIN SYNTHESIS

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I respectfully dedicate this work to
my parents and to Miss Elizabeth Longbons,
my first teacher of science.

ABSTRACT

The effects of hydroxylamine and tyrosine on homogenates of early Drosophila pupae have been studied by several criteria. Both low speed and high speed supernatants of the homogenized pupae give positive tests for hydroxamic acids after incubation with hydroxylamine. A comparison of the hydroxamic acid forming abilities of the supernatants shows that the low speed supernatant is the more active and that tyrosine increases hydroxamate formation in the low speed but not in the high speed supernatants.

Both hydroxylamine and tyrosine have definite effects on alkaline phosphatase activity in the low speed supernatant. Tyrosine causes an increase in the activity upon incubation at 0°C and 25°C. Hydroxylamine decreases the activity regardless of the temperature or the presence of tyrosine.

Both tyrosine and hydroxylamine affect the distribution of ninhydrin-positive compounds of dialyzates of low speed supernatants. Hydroxylamine effectively reduces the amount of the acidic components, but its effect is slightly modified by tyrosine. The presence of tyrosine alone increases the amount of acidic materials as compared to the untreated sample. In general, the ninhydrin-positive compounds are more sensitive to the presence of hydroxylamine than of tyrosine.

Some preliminary work on the nature of the compounds in question shows that they contain more than one amino acid. The data on the nature of these compounds is consistent with the proposition that peptidyl hydroxamates are the Fe^{+++}-positive materials.

Naturally-occurring peptides and the current understanding of protein synthesis are reviewed. The possibility that peptides per se are incorporated into the proteins in Drosophila pupae is discussed and mechanisms for this possibility are explored.

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INTRODUCTION

Peptides and Protein Synthesis

A review of the literature shows that the question of peptide intermediates in protein biosynthesis held much interest until the middle of the last decade. The decrease in interest coincides with the discovery by Hoagland (1) that each amino acid is activated by a specific enzyme in an ATP-dependent reaction. Since that time protein synthesis has generally been considered to be a process of putting single activated amino acids on a template in a stepwise fashion and then peeling off the completed polymer in a specific manner. Although a consistent scheme of protein synthesis has been worked out from the many investigations using various biological materials, the scheme appears inadequate in that no *in vitro* system has been developed which approaches the efficiency of the corresponding *in vivo* system.

The early search for peptides was not very rewarding, but, as will be shown in the next sections, there have recently been many reports of the natural occurrence of peptides in biological materials. This probably has been due to a more rigorous examination of data obtained by standard methods as well as the recent development of high resolution equipment. Yet, Chantrenne (2) made the following statement in his 1961 monograph on protein synthesis:

> And no free peptides have been found to accumulate in living cells to any considerable extent. There is at present no positive reason to think the proteolytic enzymes play an important part in protein synthesis, neither that they play any part in the process.

However, the present investigation reconsiders the question of free peptides as intermediates in protein synthesis.

Detection of Peptides in Insects

Although the latest review article (3) concerning insect biochemistry does not discuss peptides to a great extent, there is considerable evidence that peptides are a naturally occurring class of compounds in insects. Hadorn and Mitchell (4) followed peptide metabolism during the development of Drosophila melanogaster larvae and pupae and found specific peptides for both stages. Simmons (5) studied the incorporation of radioactive amino acids into a large number of components in Drosophila hemolymph. Using the techniques of paper chromatography and column fractionation he concluded that a major portion of amino acids in the hemolymph occur in bound form. This conclusion was based on the increase of ninhydrin-positive material following hydrolysis of the isolated components. Crone-Gloor (6) studied free amino acids and peptides in Drosophila eggs from the time of laying till hatching. She was able to follow three of the peptides through the developmental period, and all of these peptides seemed to be metabolically active because their concentration varied with the age of the material. Benz (7) studied two lethal mutants of Drosophila and compared them to the wild type and found amino acid and peptide differences from the wild type in both mutants. Other studies of Drosophila mutants similar to those of Benz have been reported (8,9,10).

Fox (11) studied free amino acids and peptides in Drosophila and reported the occurrence of a peptide in males that did not occur in females. This work was extended to males of sixteen different genotypes and to females of thirty-four different genotypes, and in every case the peptide difference was noted (12). Chen studied the paragonia of male Drosophila and found a ninhydrin-positive substance which corresponded to the "sex peptide" of Fox (13). Transplantation of male genital discs increased the concentration of this paragonial substance in males and caused its appearance in females.

Peptides are apparently abundant in silkworm hemolymph for the total free amino acids account for only thirty-five to fifty-five per cent of the non-protein nitrogen (14), and peptides have been isolated from silkworm pupae, but they are bound to lipid, carbohydrate, and nucleotide components. A large amount of a specific peptide has been detected in the hemolymph of the dragon-fly nymph (15). Chen and Hadorn (16) did a comparative study on three insect species, the mosquito, the flour moth and the fruit fly, and found five peptides which are characteristic for one of these species. A later study using the mosquito shows that at least three specific peptides are present (17). According to Levenbrook (18), the larval blood of the horse botfly contains at least four polypeptides. Considerable numbers of di- and tripeptides have been found in two species of beetles (19), and in these

organisms nearly all the non-protein amino nitrogen could be accounted for as amino acids, amides, and "low peptides."

Peptides and Peptide Derivatives in Biological Material other than Insects

Peptides are not characteristic of insects alone for peptides and peptide derivatives have been found in many other organisms. Several reviews on naturally-occurring peptides have been published (20,21,22,23), but none of these reviews cover the period initiated by Hoagland in the mid-1950's, and therefore do not consider peptides with respect to the current understanding of protein synthesis. A variety of neutral peptides has been isolated from cultures of Pseudomonas hydrophila (24). The concentration of the components varies with the age of the culture which suggests that they are metabolically active. Caution was taken to prevent random non-enzymatic polymerization of activated amino acids during the extraction procedure. A peptide pool has also been demonstrated in Pseudomonas saccrophila (25).

Another bacterial species, Lactobacillus casei, has been grown in basal medium complete except for the omission of one essential amino acid (26). Partial hydrolyzates of casein and bovine serum albumin were added and in ninety per cent of the cases the response to the hydrolyzates was greater than to the amino acid in question. This "strepogenin" activity has also been demonstrated in Lactobacillus delbruecki 3, Lactobacillus arabinosus, and Leuconostoc mesenteroides (27) as

well as in other microorganisms (28,29,30,31,32). One of the explanations for this effect has been that the bound amino acids are protected from endogenous decarboxylation and the amino acid is slowly released into the amino acid pool. However, Fox (33) found that the synthesis of three group A streptococcal proteins is stimulated by peptides from enzymatically digested protein. Other workers have studied the inhibition of lysine and lysine-containing peptide metabolism by thiosine and thiosyl peptides in Lactobacillus plantarum and Leuconostoc mesenteroides (34). They concluded that their data was consistent with a differential handling of the amino acid and the peptides in cellular metabolism and that similar lysine-containing peptides are apparently metabolized at very different rates.

McManus (35) investigated the peptide pool in yeast after a brief exposure to C^{14}-acetate. The specific activity of leucine isolated from peptides was much higher than that of the protein-bound leucine. Turba and Esser (36) also studied the peptides of yeast which was grown on C^{14}-acetate and found that the specific activity of the peptides increased more rapidly than that of either the amino acids or the proteins.

Kavanau (37) estimated the difference in free amino acids in protein-free extracts of fertilized sea urchin eggs before and after hydrolysis, and concluded that many peptides are present in such material. The ninhydrin-positive, 80% methanol soluble material from developing urodele eggs also

contains many peptides (38), and the distribution of this fraction changes during development--especially from the embryo to the newly hatched larvae. Peptides have even been found in the dialyzable fraction of the giant nerve fibers of the squid (39). The calf lens contains several peptides; one of these, ophthalmic acid, is present at a concentration of 20 mg/100gm of lens (40,41). Phosphopeptides have been isolated from lactating mammary gland of the rat and have been suggested as precursors for milk phosphoprotein (42), and de novo synthesis of a phosphopeptide has been realized in a goat mammary gland homogenate (43). Other materials from higher animal sources which contain peptides are suint and human sweat (44).

Synge and Wood (45) studied bound amino acids in protein-free extracts of rye grass and found that five per cent of the nitrogen in these extracts is not in free amino acids. In her studies on protein synthesis, Raacke (46,47,48) concluded that protein synthesis proceeds through various stages which include peptides and proteoses. She found that the peptide nitrogen increases at the expense of amino acid nitrogen, and then the protein nitrogen increases the expense of peptide nitrogen. Medvedev isolated cytoplasmic proteins from bean plant leaves grown in $C^{14}O_2$ (49,50,51). These proteins were subjected to complete hydrolysis by specific enzymes on one hand and to complete hydrolysis by mineral acid on the other. The uptake of the radioactivity into the same protein fraction was determined when the hydrolyzates were introduced

into new leaves, and it was found that the peptides are used more rapidly than free amino acids in protein synthesis.

Peptides linked to another group have been reported in several organisms. Nucleopeptides have been isolated from both the dialyzable and non-dialyzable fractions of Streptococcus faecalis (52,53). The stability of these compounds suggests an ester rather than a phospho-anhydride linkage. Nucleopeptides have also been reported in two other bacterial species, Escherichia coli and Bacillus subtilis (54,55,56). Adenine appears to be the only nucleobase present, but it is not known whether it exists in polyadenylic acid or as a single adenylic acid moiety. Other workers have detected carboxyl-activated peptides in bacteria as well as fungi and the liver and muscle of higher animals (57). A uridylic acid-bound peptide has been shown to occur in yeast by Gilbert and Yemm (58). Harris (59) has extended this work and found that the nucleopeptides could be trapped at the expense of protein synthesis. The concentration of the complex depends on the age of the culture; it is a maximum in logarithmic growth phase, but then decreases as the culture ages (60). The nucleopeptides appear to be synthesized by the yeast cell in excessive amounts in early growth, but they turn over rapidly during logarithmic growth. The nucleotide moiety is also uridylic acid although five compounds contained the dinucleotide, adenyl uridylate (61,62,63). These nucleopeptides have been separated and purified by ion-exchange chromatography and most of the compounds contain

the same amino acids, but no sequence studies have been done (64). Koningsberger, et al. (65,66), have also identified carboxyl-activated peptides in yeast, and in this case adenosine and cytosine were the nucleoside moieties. These workers also found that peptides could be activated by the soluble protein fraction of yeast (67) and seven to nine amino acids were found in these activated peptides. Nucleopeptides in yeast have also been the subject of an extensive investigation by Klienauf (68). Hase found sulfur-containing nucleopeptides in yeast and Chlorella (69,70,71,72,73), and discovered that the concentration of these nucleopeptides in synchronized cultures increases just before cell division. Haberman (74) has found peptides associated with RNA from several sources--yeast, ascites tumor cells, mouse liver and mouse brain. Peptides that are associated with nucleotides have also been reported in rat liver by two groups of workers (75,76) as well as in rabbit liver (77). The hepatic nucleopeptides of rabbit occur most abundantly in the microsomal supernatant fraction, but they were also found in TCA extracts of the microsomal, mitochondrial and nuclear fractions. Uridine diphosphoglucose was the nucleotide moiety of four of these compounds (78), while uridylic, guanylic, and cytidylic acids assumed this function in the remaining ones which were studied extensively.

Szafranski, et al. (79,80), have made a fairly thorough study of a class of nucleopeptides in guinea pig liver. The nucleotide moiety appears to be a polymer since all the bases

can be detected after hydrolysis of a purified sample (81,82). The nucleopeptides differ from one another by their nucleotide as well as by their peptide moieties. The same workers have found similar nucleopeptides from the nuclei of guinea pig liver (83). The enzymatic activation of peptides by pH 5 enzymes from pigeon liver has been demonstrated (84); the amount of hydroxamate was measured quantitatively and the products were identified chromatographically. Nine synthetic di- and tripeptides were capable of being activated in this system and seven of them gave greater yields of hydroxamate than the amino acid leucine. Glutathione has been found to participate in an activation reaction with enzymes from mouse and human muscle (85).

Peptides occur in nature in complexes with various other groups, but they do not seem to be of the phospho-anhydride type analogous to the amino acid adenylate of the protein synthesis scheme. The best characterized peptide complex is a component of bacterial cell walls. The peptide is linked to the carboxyl group of lactic acid which is bound through an ether group to N-acetylglucosamine which is in turn linked to uridine diphosphoglucose (86). A nucleopeptide which does not react with hydroxylamine has been isolated from beef liver (87), and has been identified as a 3', 5' adenosine diphospho-peptide which also contains a third phosphate group. The peptide moiety contains six ninhydrin-positive components and three of them are amino acids not common to proteins; namely, β-alanine, cysteic acid, and taurine. King salmon liver

contains adenyl succinate groups linked to peptide through a phosphate-sulfate group (88). Another unusual type of complex is a coenzyme A bound peptide or group of peptides that has been detected in rat liver (89).

There seems to be little doubt that peptides and peptide derivatives are normal constituents of microorganisms, plants, and animals, however there have been several reports of the failure to find peptides in natural sources (90,91, 92,93). In this respect, Deane and Truter (44) give some very good advice when they point out that many peptides have chromatographic behavior in two-dimensional systems that is identical to amino acid behavior and that incorrect assignment of structure is possible in the absence of any other tests. The minimum test for the absence of peptides in a ninhydrin-positive component is a single component upon chromatography before and after hydrolysis. The presence and abundance of peptides in some biological materials and the absence of peptides in others is an anomaly that must be considered in the attempt to understand the overall picture of cellular metabolism.

The role of peptides in biological systems has been discussed for a number of years and the majority of investigators concerned with protein biosynthesis do not seem to consider peptides as intermediates (90,91,94,95,96,97). This conclusion is based on the failure to find peptides and the fact that known peptides have other functions as anti-biotics, cofactors, and hormones. The literature

review presented here shows that there are a great many peptides in nature for which a function has not been determined.

The Current Understanding of Protein Biosynthesis

The current knowledge and understanding of protein biosynthesis present the following scheme. Amino acids are activated by specific enzymes in a reaction that involves adenosine triphosphate and results in an enzyme-bound amino acid adenylate complex (1,98,99). Several of these specific enzymes have been partially purified (100,101,102,103,104,105). The reaction exchanges pyrophosphate and requires stoichiometric amounts of enzyme unless the cell sap fraction containing soluble ribonucleic acid is present. The reaction is formulated in two steps as follows:

1. Enzyme + ATP $\rightleftharpoons$ Enzyme-ATP

2. Enzyme-ATP + amino acid $\rightleftharpoons$ Enzyme-amino acid adenylate + PP

The activated amino acid is then transferred to soluble ribonucleic acid molecule in a reaction catalyzed by the same enzyme. The sRNA molecules are specific for each amino acid for increasing the concentration of added amino acid results in the attainment of a saturation level (106). The specificity of the sRNA molecules as acceptors for different amino acids is not related to molecular weight (107), and it appears that the specificity must reside in its nucleotide composition. However, the terminal trinucleotide sequence at the 3' hydroxyl end of the RNA chain has been identified in several organisms as pCpCpA (108,109,110).

Adenosine-amino acid esters have been identified as products of a RNAase digest of charged sRNA's (109,111), while borate ions (108) and periodate oxidation (111) inhibit the amino acid-sRNA combining reaction. These facts suggest that the amino acid-sRNA bond is an ester linkage of the carboxyl group of the amino acid with the 2' or 3' hydroxyl group of the ribose moiety of the terminal adenosine residue. In a specialized system phenylalanine-sRNA has been shown to be an intermediate in the cell-free synthesis of polyphenylalanine (112).

The amino acid-sRNA linkage has high energy character as is shown by the equilibrium constant for the amino acid-sRNA reaction (113,114):

Amino acid adenylate-enzyme + sRNA $\longrightarrow$ amino acid-sRNA + AMP

For L-valyl-ribonucleic acid formation the equilibrium constant is 0.32 (115). Raacke (113) has pointed out that this type of ester reacts with neutral and slightly alkaline hydroxylamine; this also suggests that the amino acid-sRNA contains an "active acyl" group.

The amino acyl-sRNA molecules then transfer the amino acids to the ribosome. Presumably the ribosome is first "conditioned" by messenger RNA from the nucleus. Numerous reports of a RNA species whose synthesis is dependent on DNA have appeared in recent months (116,117,118,119,120,121), and naturally-occurring DNA-RNA complexes have also been reported (122,123,124). These experiments have led to the conclusion

that there is a species of RNA whose synthesis is DNA-directed and whose base sequence is determined by the sequence of the bases in the directing DNA. Hurwitz and Furth (125) have briefly summarized the role of messenger RNA and have suggested how the messenger RNA molecule might alter the topography of the ribosome. Figure 1 is a diagram similar to the one presented by Hurwitz and Furth for the synthesis of a peptide chain on the ribosome.

More recently the adaptor hypothesis has been tested experimentally (126). The adaptor hypothesis of Crick (127) and Hoagland (128) holds that the position of an amino acid is determined by hydrogen bonding between the messenger RNA and a complementary nucleotide sequence in the sRNA molecule carrying the amino acid. CySH-sRNA^{CySH} was altered by reductive desulfhydration with Raney Nickel to give Ala-sRNA^{CySH}. (The superscript refers to the type of specific sRNA before treatment with the reducing agent.) The Ala-sRNA^{CySH} was incorporated into polypeptide under the direction of poly UG which normally stimulated the incorporation into polypeptide of cyteine but not alanine. The interpretation of these facts is that the amino acid does not participate in coding after it is attached to its sRNA molecule. Implicit in this statement is the suggestion that in the living cell there is no alteration of amino acids once they have been bound to their specific sRNA's, and that these sRNA-bound amino acids must be protected from the enzymes that normally alter them in cellular metabolism for alteration of sRNA-bound amino acids would result in the formation of nonsense proteins.

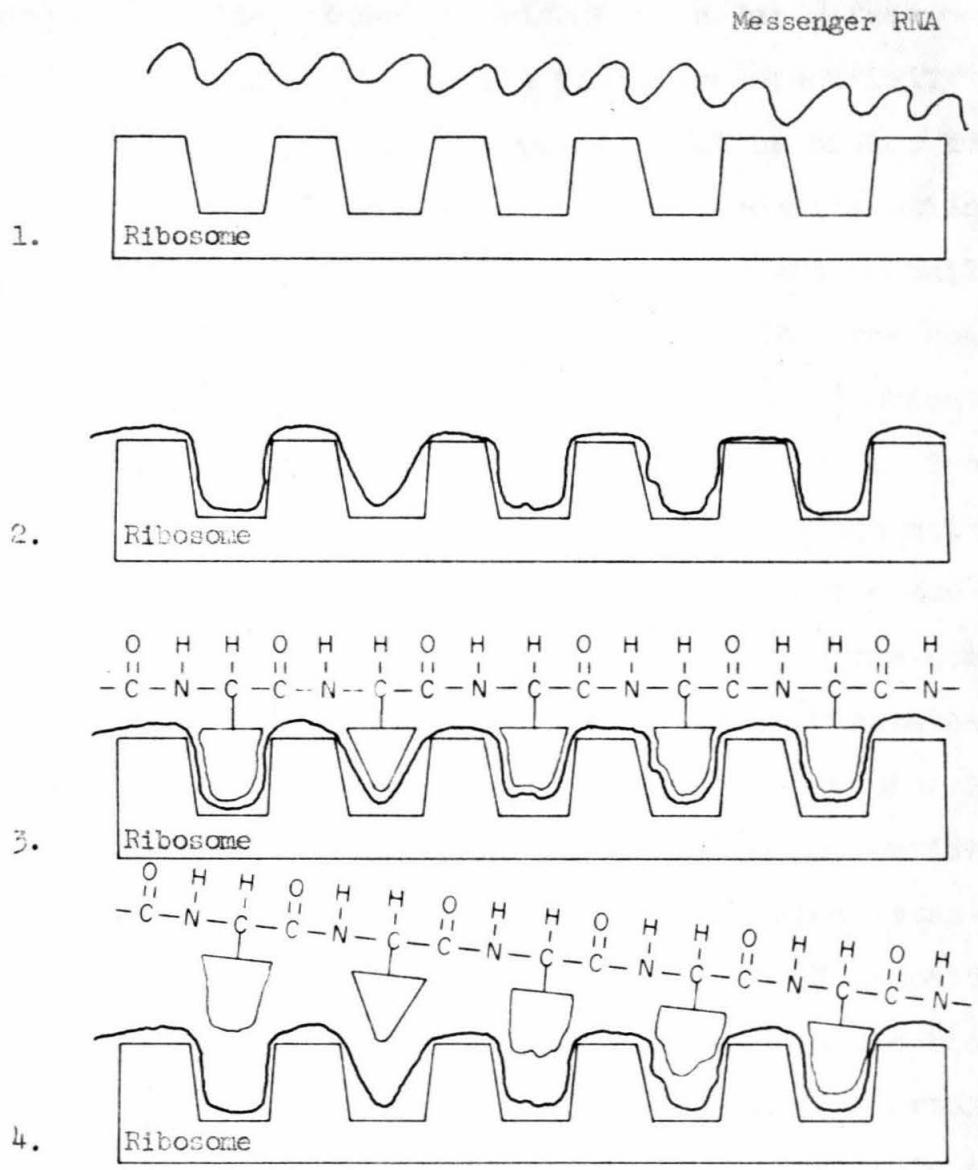


Figure 1. Synthesis of a peptide chain on the ribosome from amino acyl RNA's (after Hurwitz and Furth) (125).

1. Messenger RNA and ribosomal surface.
2. Modification of ribosomal surface by messenger RNA.
3. Assemblage of amino acids of ribosome via sRNA derivatives.
4. Removal of finished peptide chain.

Basis for Present Work

The existence of a large number of peptides in Drosophila larvae and pupae, as mentioned above, lend this organism to a study of peptide metabolism with particular reference to protein biosynthesis. Drosophila pupae are essentially closed systems in which the larval protein must be broken down and reassembled into adult protein. Energy considerations suggest that preformed peptides would be attractive building blocks for the formation of adult protein for the energy normally expended in the synthesis of a peptide bond would be saved for every intact peptide linkage that is incorporated into new protein. Non-random labelling of amino acids incorporated into ovalbumin suggests that intermediate compounds do exist between the free amino acid pool and the completed protein molecule (97). If free peptide intermediates that are "on the way up" do exist, they must participate in some sort of activation reaction. The reaction to consider first is that of the formation of peptidyl acyl adenylates--essentially the amino acid activation reaction. If peptides can be activated, then their possible role in protein biosynthesis can be explored. This experimental work was undertaken with recognition of the meager understanding of the role of peptides in cellular metabolism.

METHODS AND MATERIALS

Culture of Drosophila Pupae

Pupae of the Oregon R wild type were obtained by the following method. Several thousand flies were housed in a lucite box which measured 46 x 46 x 31 cm, and the population was maintained at a level to give synchronized cultures from a two-hour laying period. The flies were fed on a corn meal-agar-molasses medium in open Petri plates with a spot of a thick suspension of Baker's yeast in a two per cent sucrose solution on top of the solid medium. The feeding plates were changed two hours before the collection of the eggs was begun; the egg collection consisted of putting into the cage half-pint milk bottles containing the corn meal-agar medium topped with yeast. After the desired laying period the flies were shaken out and the bottles stoppered. Water was added to keep the yeast soft, and, if the strength of the culture warranted, more of the yeast suspension was added on the third day. The laying period was usually one and one-half to two hours, but, if the experiment required five or more grams of pupae, two successive collections of twelve bottles each were used. After pupation was complete at 120 hours (25°C constant temperature), the inside walls of the bottles were sprayed with water from an atomizer. The water treatment loosened the pupae, and they could be scraped from the bottles without damage. The pupae were washed thoroughly in tap water and spread on paper towels to dry, and any larvae

that were present were removed. Checks on this method of preparation showed that one hundred per cent of the pupae hatched into adult flies. A good collection yielded between four and five grams of pupae from twelve bottles.

Homogenization and Centrifugation

After the pupae were weighed they were homogenized in a glass cone grinder driven by an electric stirring motor. Two milliliters of cold tap water were used for each gram of pupae. The homogenization process required about three minutes per gram of pupae and was carried out in an ice-salt bath at -2°C. The resulting homogenate was extremely viscous, but could be drawn up in a machine-made capillary pipette. The homogenate was centrifuged at 8,000 rpm for thirty minutes in a Model L Spinco ultracentrifuge using a precooled 40 or 40.3 rotor.

The centrifuged preparation consisted of a large brown sediment and a supernatant. The sediment contained pupal cases and cellular debris with a grayish-white layer on top while the supernatant was amber colored and slightly cloudy, especially near the surface of the sediment. A layer of fat floated on top of the supernatant and was partially removed with a cold spatula. The supernatant was removed with a cold Pasteur pipette and was transferred to conical graduated tubes in an ice bath. All of these operations were conducted in a manner to avoid a significant deviation from 0°C.

Microsomal supernatants were prepared from the 8,000 rpm supernatants by centrifuging at 40,000 rpm (105,000 x g) for two hours in the Model L ultracentrifuge.

Preparation of Low-salt Hydroxylamine and Hydroxamic Acid Assay

Low-salt hydroxylamine was prepared from hydroxylamine hydrochloride by a modification of the method of Beinert (129). Hydroxylamine hydrochloride (Mallincrokdt) of ninety-six per cent purity was dissolved in methanol (11.7 gm/100ml) and enough methanolic potassium hydroxide (15 gm/40 ml) was added to give a pH of 7.0 when diluted eight-fold with distilled water before the pH measurement. The potassium chloride was filtered from the methanolic solution and the filtrate was concentrated with a gas ballast pump equipped lyophilizer. The concentrated solution was then diluted to give a six molar solution and stored in the freezing compartment of a refrigerator. One preparation was made with pH 8.4, 0.2 M tris buffer for enzymatic studies.

Hydroxamate formation was assayed by adding 0.7 ml. of the incubation mixture (8,000 rpm supernatant plus various additions) to 0.2 ml of a twenty per cent trichloroacetic acid solution and mixed by shaking. These were centrifuged for thirty minutes at 12,000 rpm at 25°C in a Servall RC-2 automatic superspeed centrifuge. The supernatants were decanted or drawn off with a capillary pipette; 0.1 ml of 5% $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl was added to 0.4 ml of the supernatant. These solutions were mixed thoroughly and read in a Model 11MS

Cary spectrophotometer at 540 mμ. A standard curve which was a straight line between 0 and 1.0 moles of hydroxamate per milliliter was obtained with leucine hydroxamate (Sigma). 1.0 mole of hydroxamate per milliliter gave an optical density of 0.5.

Alkaline Phosphatase Assay

The 8,000 rpm supernatant was used as the source of alkaline phosphatase and was assayed by the method of Mitchell (130) which consists of adding fifty microliters of the enzyme preparation to 0.4 ml of substrate in buffer and then following the initial rate of the reaction at 400 mμ in the Cary spectrophotometer. A 2 mg/ml solution of p-nitrophenylphosphate diluted thirtyfold in pH 8.4, 0.2 M tris was used as the substrate in buffer solution. Enzymatic activity is expressed as the change in optical density per ten minutes of reaction time.

Chromatography and Electrophoresis

Ascending chromatography was done in battery jars equipped with a support hanging through a hole in the glass cover. The two solvent systems used were propanol-ammonia and butanol-acetic acid-water. Propanol-ammonia is made from two parts of n-propyl alcohol and one part of three per cent ammonium hydroxide solution. Chromatography in this solvent was done at room temperature. The butanol-acetic acid-water solvent is made from four parts n-butanol, one part glacial acetic acid, and five parts water. Since

this solvent is a two phase system it was used in a constant temperature room at 25°C.

High voltage electrophoresis was carried out in a Gilson Electrophorator at 2,000 volts for one hour on Whatman 3MM paper which measured 46 x 56 cm. The solvent was pyridine-glacial acetic acid-water (1:10:289) adjusted to pH 3.7. Other high voltage electrophoresis was done at 5,000 volts for ninety minutes with 12 x 41.5 inch sheets of Whatman 3MM paper and with eight per cent formic acid as the solvent.

Dowex 50w-x2 columns of known amino acid hydroxamates and hydroxylamine-treated Drosophila 8,000 supernatants were run using a pyridinium formate elution system. The column bed was either 40 x 1 cm or 155 x 1 cm. Fractions of five milliliters were collected, lyophilized, and dissolved in 100 microliters of glass distilled water. Three microliters of each sample was spotted on Whatman No. 1 paper and chromatogrammed. Hydroxamates were located by dipping the chromatogram in 5% $FeCl_3 \cdot 6H_2O$ in butanol. If the solvent was propanol-ammonia the chromatogram was then placed in a cylinder jar containing a beaker of acetic acid. This lowered the pH of the moisture on the chromatogram and the hydroxamate-iron complex formation proceeded to give visible purple spots. If the solvent was butanol-acetic acid-water, no such treatment was necessary. Ninhydrin reacting material was located by dipping the chromatogram in a solution of 2.5% ninhydrin in acetone and air drying before heating at 100°C for ten minutes.

Amberlite 120 columns were run on a Spinco Model 120 Amino Acid Analyzer. The buffer system employed was 0.20 N citrate, pH 3.25; 0.20 N citrate, pH 4.25; and 0.35 N citrate, pH 5.28 followed by 0.20 N sodium hydroxide to "chase" the column. The buffers were prepared according to the instruction manual for the Model 120 (131). Ten per cent of the sample was run through the ninhydrin bath and recorded automatically while the remaining ninety per cent was collected in a Gilson fraction collector by means of a stream splitter on the Model 120. Hydroxamate color was determined by the usual procedure on the collected fractions. Other amberlite 120 columns were run on the Model 120 with all the sample going to the ninhydrin bath.

RESULTS

Hydroxamate Formation

If the 8,000 rpm supernatant of homogenized Drosophila pupae is incubated with low-salt hydroxylamine at a final concentration of 1.0 M, a series of timed aliquots reveals an increase of absorption at 540 mμ upon the addition of ferric ion. The hydroxamate forming reaction is dependent on temperature; if the reaction mixture is incubated at 0°C and then raised to 25°C, the rate of hydroxamate formation increases at the time of temperature change (Figure 2, curve A).

Mitchell (130) has found that radioactive tyrosine behaves in an unexpected manner when added to pupal homogenates; it is incorporated very rapidly into several ninhydrin-positive components at 0°C. Added tyrosine also has a definite and somewhat unexpected effect on hydroxamate formation by such homogenates. Curve B of Figure 2 shows that if tyrosine is added to the reaction mixture, the rate of hydroxamate formation is greatly increased. However, raising the temperature of the tyrosine-containing mixture does not increase the rate of hydroxamate formation after an hour's incubation at 0°C.

If the 8,000 rpm supernatant is centrifuged at 40,000 rpm to sediment the microsomes, the resulting microsomal supernatant can also carry out the hydroxamate forming reaction. Figure 3 shows three curves; A and C are without

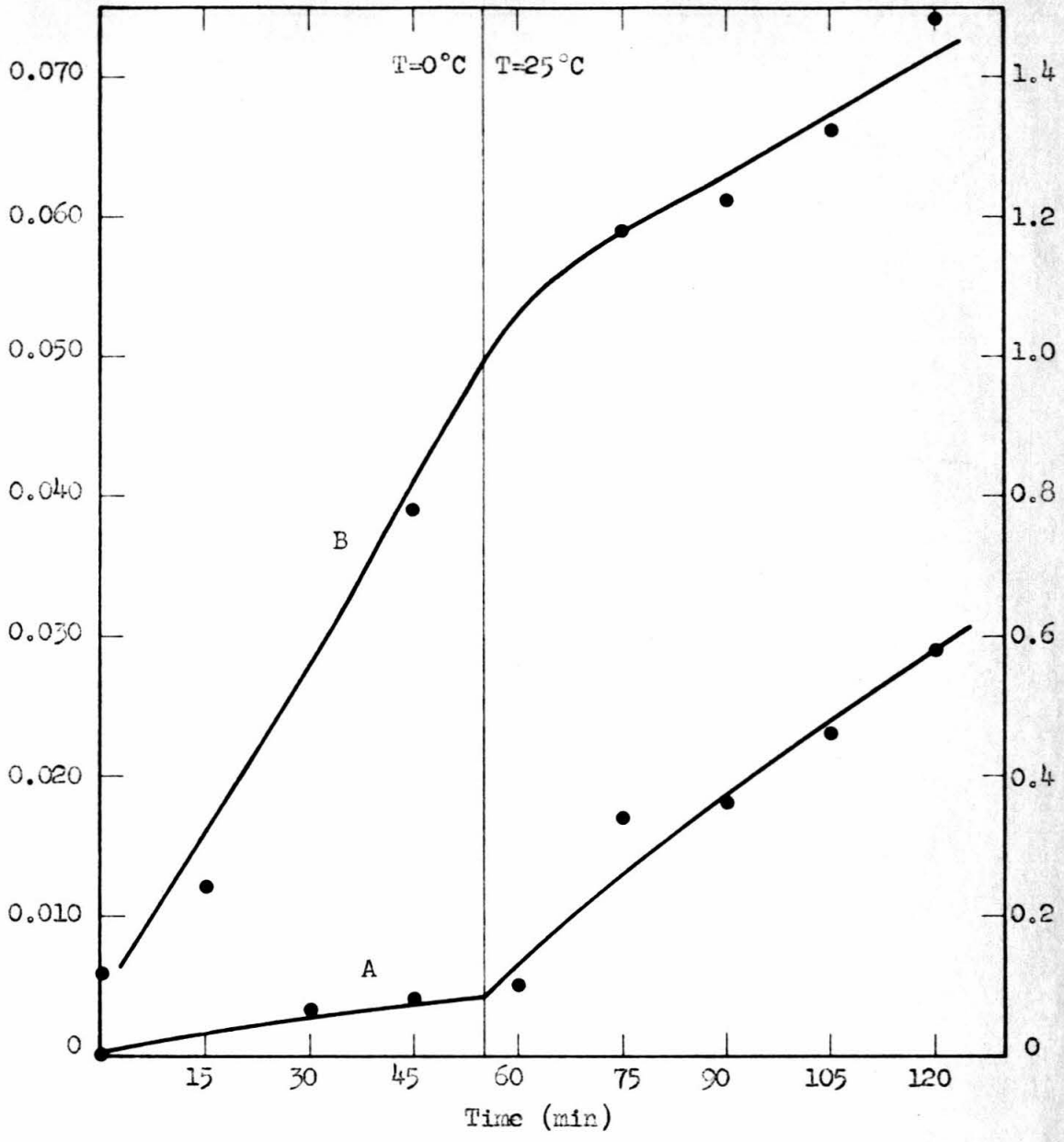


Figure 2

Hydroxamate formation by 8,000 rpm supernatant of 122 ± 2 hour Drosophila pupae. Left scale: optical density. Right scale: μmole hydroxamate/ml reaction mixture. (8,000 rpm = 4220 x g_{ave})

A	B
4.0 ml 8,000 supernatant	4.0 ml 8,000 rpm supernatant
3.0 ml 6M NH_2OH	3.0 ml 6M NH_2OH
8.0 ml H_2O	0.6 ml tyrosine solution
	7.4 ml H_2O

Final concentration of tyrosine was 2 x 10^{-4} M. Duplicate 0.7 ml aliquots taken at indicated times. Details of assay are given in Methods and Materials Section.

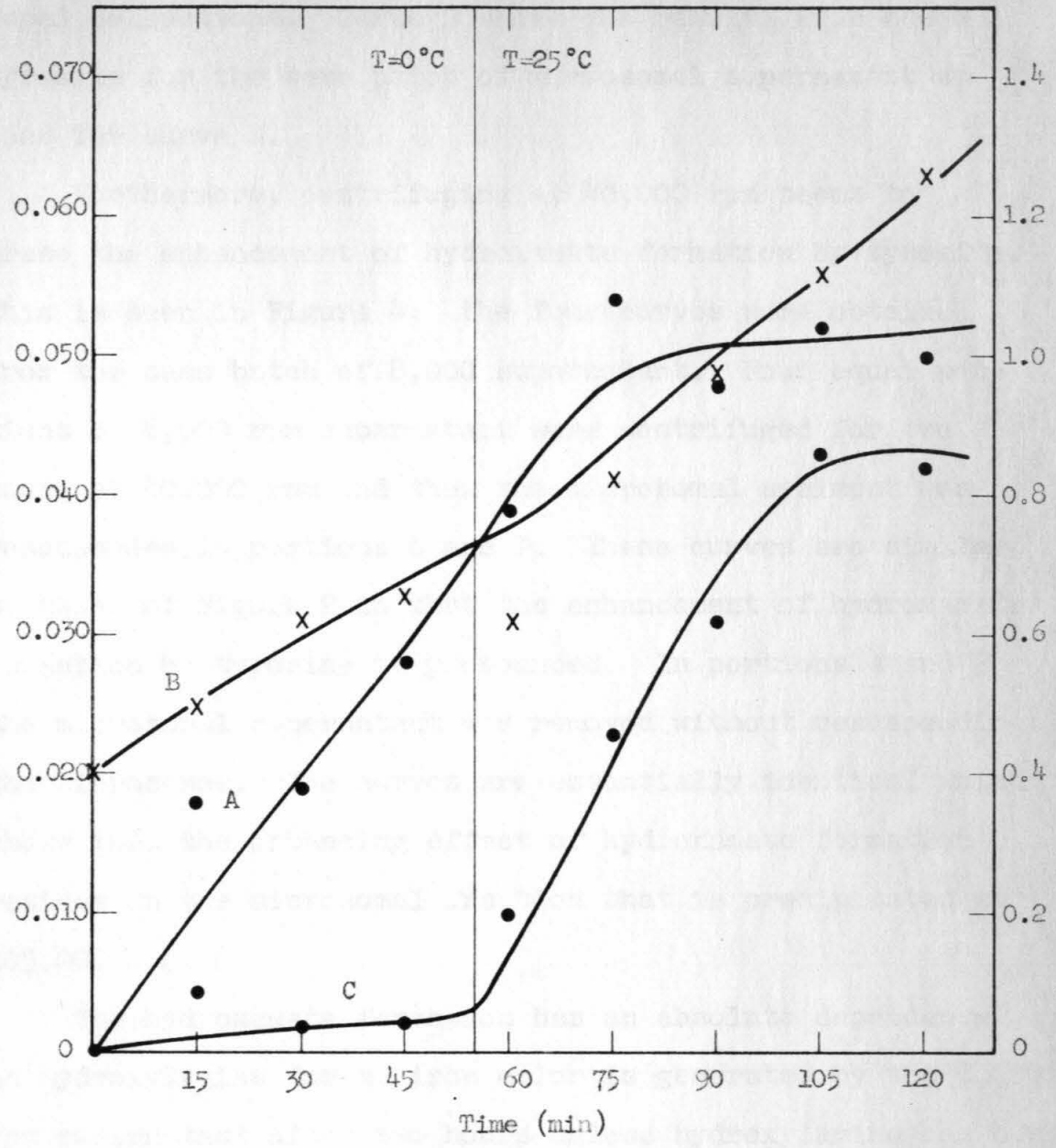


Figure 3

Hydroxamate formation by microsomal supernatant of 122 hour Drosophila pupae. Left scale: optical density. Right scale: µmole hydroxamate/ml reaction mixture.

A	B	C
9.0 ml micr. super	9.0 ml micr. super	11.0 ml micr. super
1.8 ml 6 M NH_2OH	1.8 ml 6 M NH_2OH	3.0 ml 6 M NH_2OH
0.36 ml H_2O	0.36 ml tyrosine	1.0 ml H_2O

Final concentration of tyrosine in B was 1.6×10^{-4} M. 0.7 ml aliquots taken at indicated times. Details of assay given in Methods and Materials Section.

added tyrosine, but done on different days with different pupal collections. Curve B shows the results with added tyrosine for the same batch of microsomal supernatant as used for curve A.

Furthermore, centrifuging at 40,000 rpm seems to erase the enhancement of hydroxamate formation by tyrosine. This is seen in Figure 4: the four curves were obtained from the same batch of 8,000 supernatant. Four equal portions of 8,000 rpm supernatant were centrifuged for two hours at 40,000 rpm and then the microsomal sediment was resuspended in portions C and D. These curves are similar to those of Figure 2 in that the enhancement of hydroxamate formation by tyrosine is pronounced. In portions A and B the microsomal supernatant was removed without resuspending the microsomes. The curves are essentially identical which shows that the enhancing effect of hydroxamate formation resides in the microsomal fraction that is precipitated at 105,000 x g.

The hydroxamate formation has an absolute dependence on hydroxylamine for no iron color is generated by the 8,000 rpm supernatant after two hours unless hydroxylamine has been added. An ATP-generating system of creatine phosphate and creatine phosphokinase also does not increase hydroxamate formation in these supernatants. Furthermore, other amino acids do not give the enhancement of hydroxamate formation as does tyrosine. Leucine, glutamic acid, alanine, and

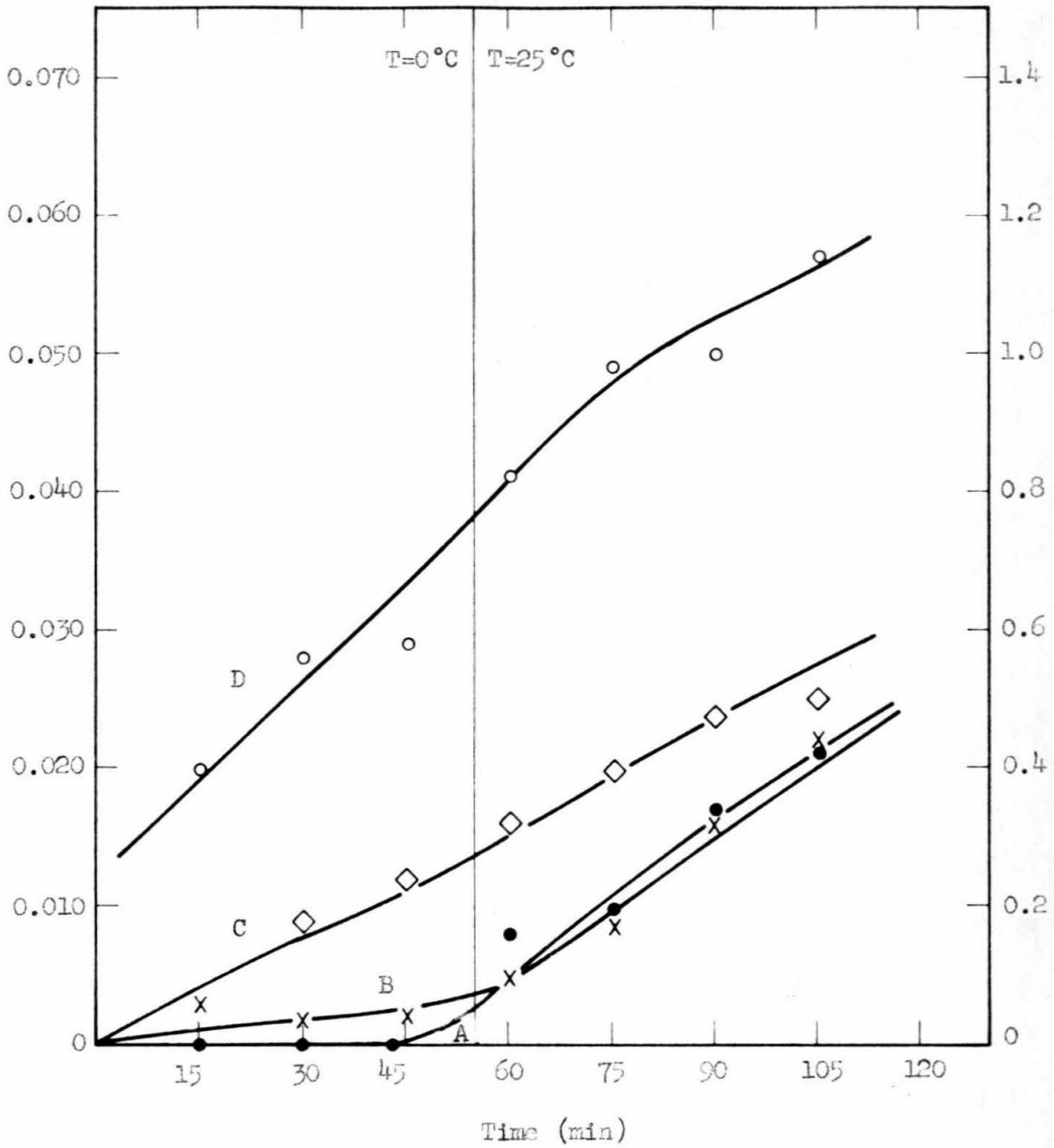


Figure 4

Comparison of hydroxamate formation by 8,000 rpm and microsomal supernatants. Left scale: optical density. Right scale: μmole hydroxamate/ml reaction mixture.

A	B	C	D
3.0 ml 40,000	3.0 ml 40,000	3.0 ml 8,000	3.0 ml 8,000
1.4 ml NH_2OH	1.4 ml NH_2OH	1.4 ml NH_2OH	1.4 ml NH_2OH 6M
	0.24 ml tyrosine		0.24 ml tyrosine
2.6 ml H_2O	2.36 ml H_2O	2.6 ml H_2O	2.36 ml H_2O

Final concentration of tyrosine was 2×10^{-4} M. 0.7 ml aliquots at indicated times. Details of assay given in Methods and Materials Section.

arginine have been tested under identical conditions and at the same concentration as used with tyrosine, but, on the other hand, none of them show any inhibitory effect.

Alkaline Phosphatase

Mitchell (130) has studied the activity of alkaline phosphatase in low speed supernatants of pupal homogenates and has found that the enzymatic activity of these supernatants is greater at 25°C than at 0°C. If tyrosine is added to the incubation mixture, the alkaline phosphatase activity is also increased. This effect is most pronounced after the temperature has been raised. The tyrosine effect has been confirmed in this work and is shown in Figure 5, curves A and B.

The addition of hydroxylamine has a curious effect on the enzymatic activity of Drosophila supernatants; the activity increases slightly at the beginning of the incubation at 0°C, but starts to decline soon thereafter. The activity continues to decrease and does not increase even if the temperature is increased to 25°C. This hydroxylamine effect on alkaline phosphatase activity is shown by curves C and D in Figure 5.

Figure 5 is a composite of two experiments; it shows the general relationship of the enzymatic activity with respect to tyrosine, hydroxylamine and temperature. The alkaline phosphatase activity varies considerably from preparation to preparation, and the variations are noticed

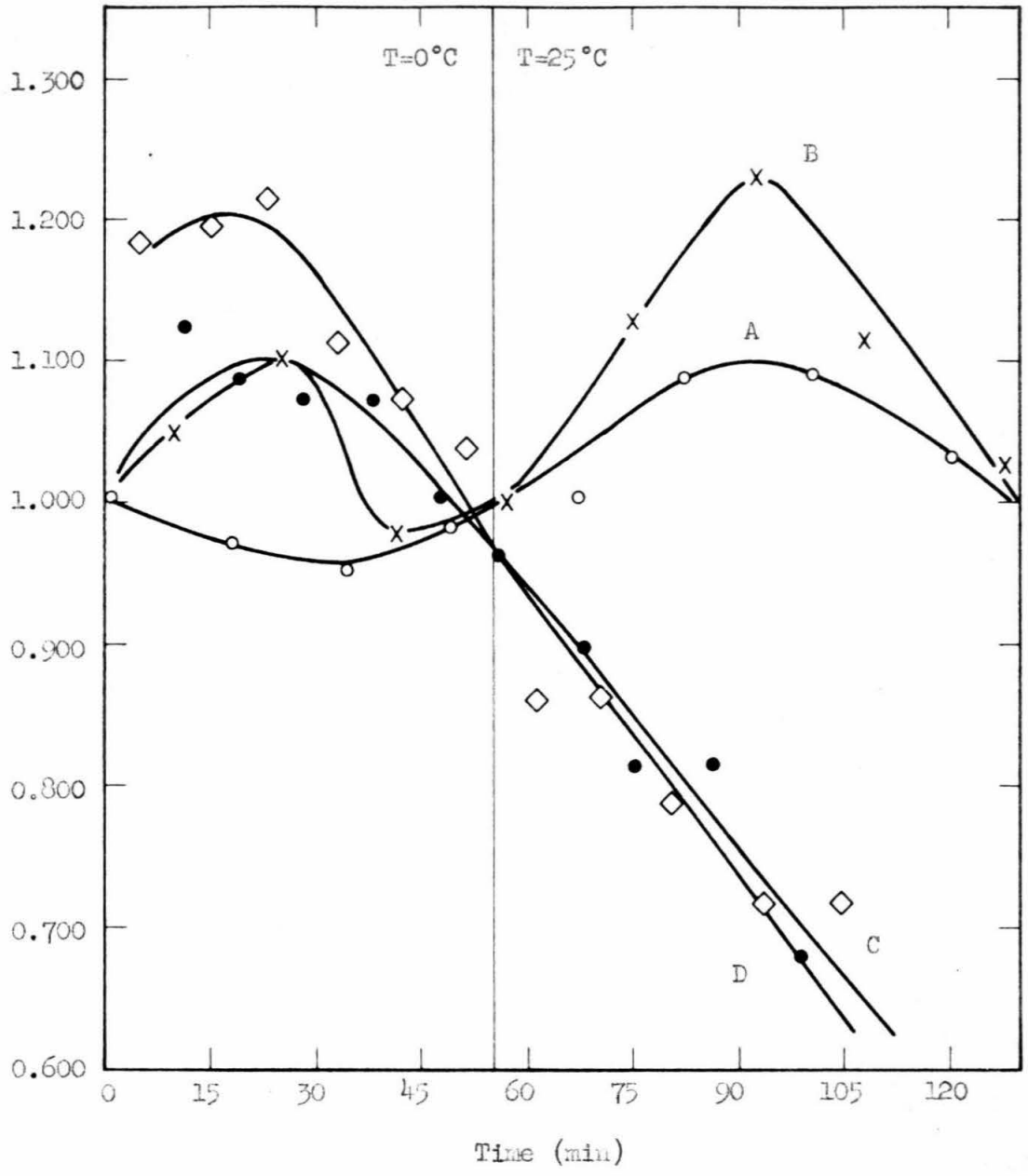


Figure 5

Alkaline phosphatase activity. Ordinate scale: change in absorbance per ten minutes per fifty microliters of enzyme preparation.

A	B	C	D
1.0 ml 8,000	1.0 ml 8,000	1.0 ml 8,000	1.0 ml 8,000
0.04 ml H_2O	0.04 ml tyrosine	0.04 ml H_2O	0.04 ml tyrosine
0.20 ml H_2O	0.20 ml H_2O	0.20 ml NH_2OH	0.20 ml NH_2OH

Final concentration of NH_2OH = 1 molar. Final concentration of tyrosine = 1.6×10^{-4} molar. Details of assay given in Methods and Materials Section.

with respect to the total enzyme activity, the percentage increase upon raising the temperature, and the percentage increase upon the addition of tyrosine. However, the general pattern shown in Figure 5 is representative. The only other amino acids whose effect on alkaline phosphatase has been determined are glutamic acid and leucine, and these amino acids do not result in any increase or decrease in the activity.

Effect of Tyrosine and Hydroxylamine on Ninhydrin-positive Compounds

The previously described experiments show that both tyrosine and hydroxylamine have a measurable effect on hydroxamate formation and alkaline phosphatase activity in Drosophila homogenates. It is of primary importance to ascertain what compounds or classes of compounds are being affected by tyrosine and hydroxylamine. Since ninhydrin-positive compounds are proposed to be intimately involved in the problem being investigated, Drosophila homogenates that had been treated with (I) neither (II) hydroxylamine (III) tyrosine (IV) both tyrosine and hydroxylamine were run through the Model 120 Amino Acid Analyzer. The resulting ninhydrin-positive traces are shown in Figure 6. Since the same amounts of material were used for each column, the traces should be quantitatively comparable.

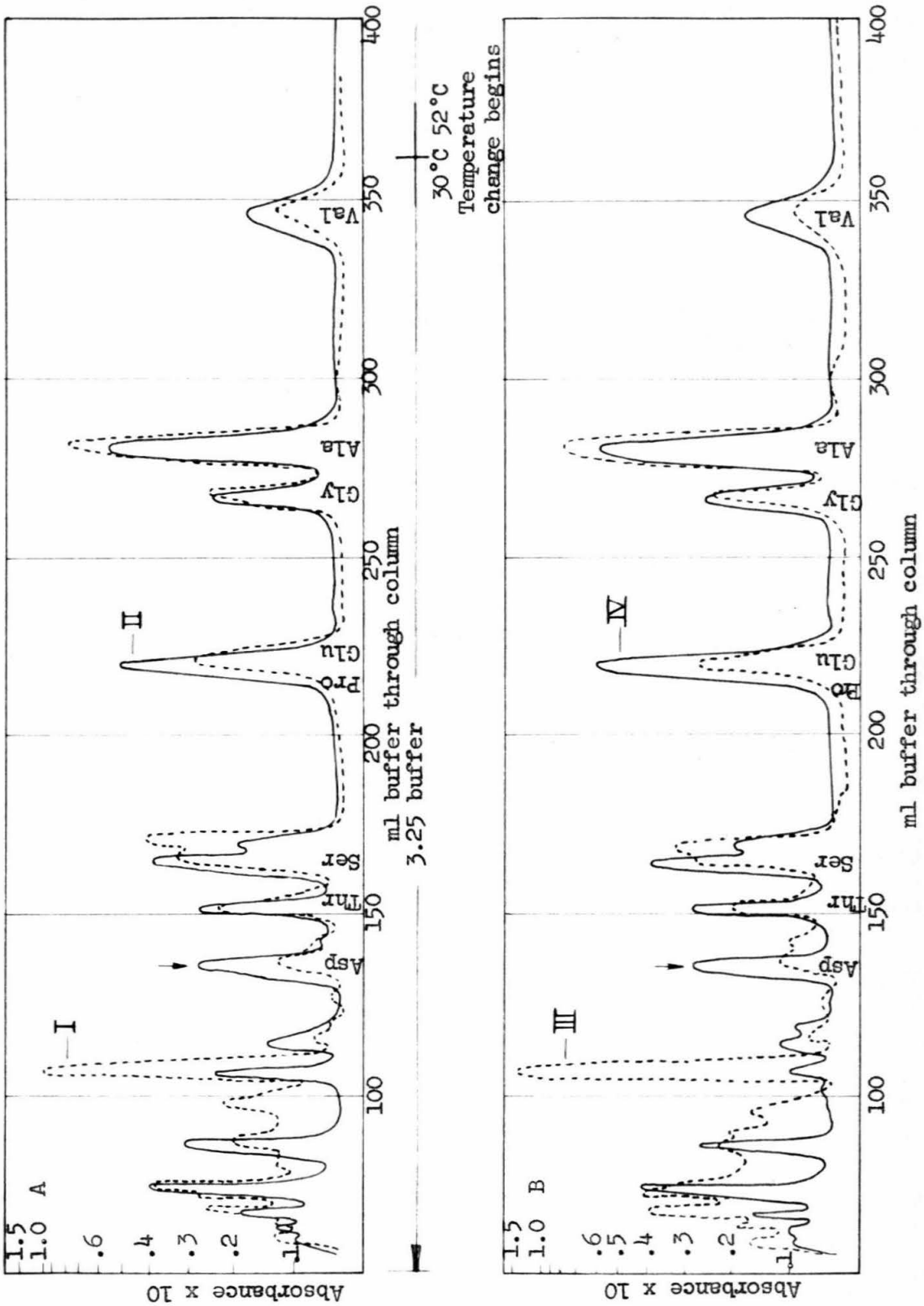

Absorbance x 10
1.5
1.0
.6
.4
.3
.2
A
B
I
II
III
IV
Asp
Thr
Ser
Pro
Glu
Gly
Ala
Val
100
150
200
250
300
350
400
ml buffer through column
3.25 buffer
30°C 52°C
Temperature
change begins

Figure 6

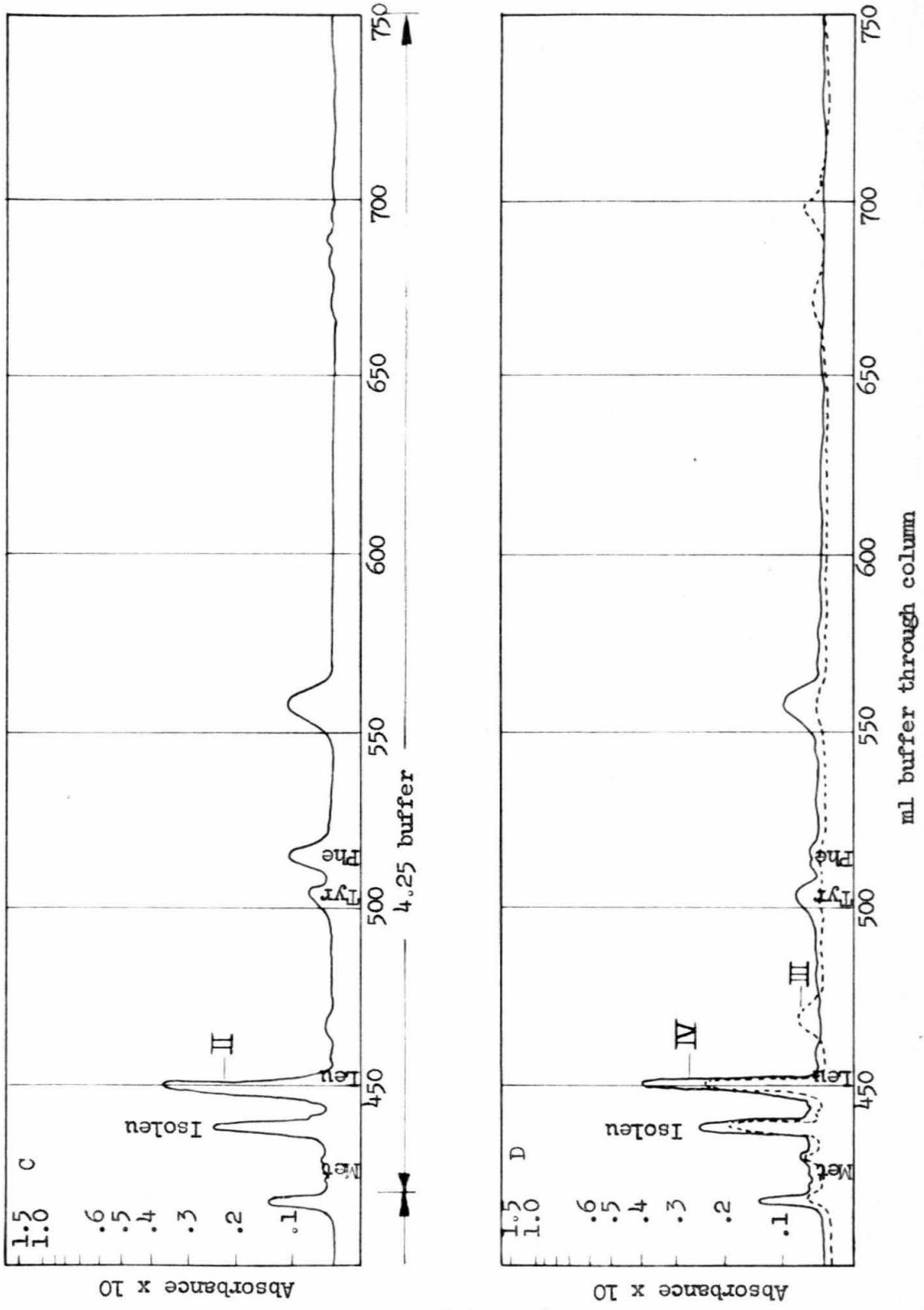


Figure 6 (cont.)

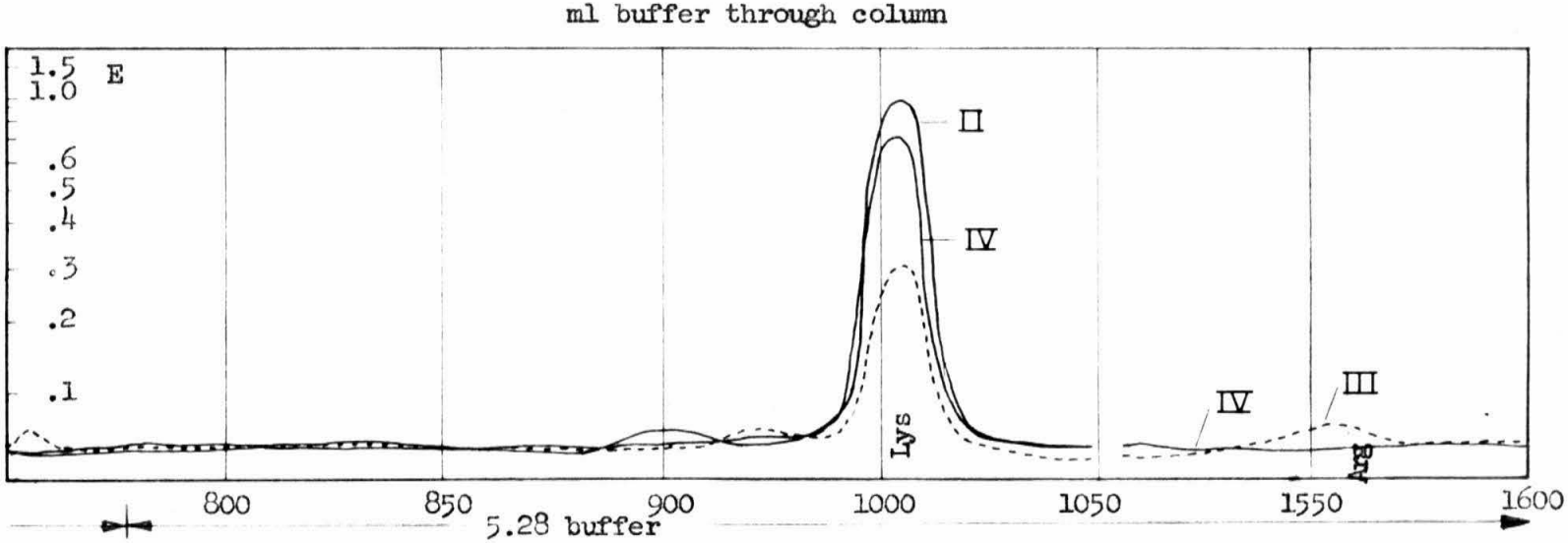


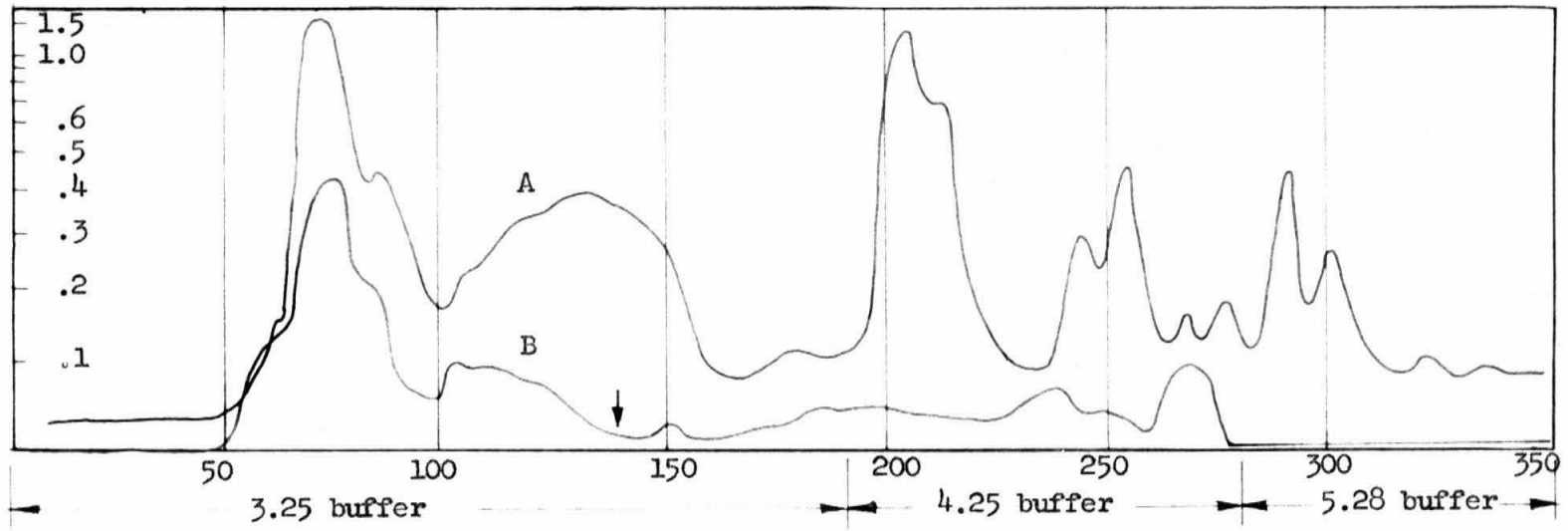
Figure 6 (cont.)

Ninhydrin positive traces of Drosophila 8,000 rpm supernatants treated with (I) nothing (II) hydroxylamine (III) tyrosine (IV) both tyrosine and hydroxylamine. 24.8 grams of 122 ± 2 hour Drosophila pupae were homogenized in 49.6 ml of cold tap water. The homogenate was centrifuged at 8,000 rpm for 30 minutes and 37.6 ml of supernatant were recovered. The supernatant was divided into four equal parts of 9.4 ml and tyrosine, hydroxylamine, or both were added. All tubes were diluted to 15 ml. The final concentration of tyrosine was 2 x 10^{-5} M, and the final concentration of hydroxylamine was approximately 1.0 M. After incubation at 25°C for two hours, the samples were dialyzed against 200 ml of distilled water for 12 hours. The dialyzates were lyophilized and dissolved in 8 ml of distilled water. Each sample was run through a Dowex 50w-x2 column which was in the formate form and had been equilibrated with pH 2.37 pyridinium formate (made by mixing 335 ml of 8.8% formic acid with 51 ml of 10% aqueous pyridine). The column was flushed with 50% aqueous pyridine and the eluate collected, lyophilized, and dissolved in 10 ml of distilled water. Only II and IV gave a positive reaction with ferric ion, but all of them contained the same amount of ninhydrin positive material. One ml of each of these samples were lyophilized and dissolved in 2.5 ml of 2.20 citrate buffer (0.20 N in sodium). Two ml of each was put on the Amberlite 120 column. Thus the material put on the column was from about 0.5 gm of pupae. The curves plotted are the absorbance at 570 mμ. The termination of trace I was due to operational difficulties.

Isolation of Fe^{+++}-positive Material

If known amino acid hydroxamates are subjected to ion-exchange chromatography on a forty-five x one centimeter column of Dowex 50w-x2 resin with a pyridinium formate gradient, the hydroxamates begin to emerge after 200 ml of buffer have passed through the column. If 8,000 rpm supernatant is incubated with hydroxylamine for two hours at 25°C and the dialyzable portion subjected to the same ion-exchange treatment, Fe^{+++}-positive material emerges after only twenty-five ml of buffer have been collected. Three hundred and fifty milliliters of eluate produced by stepwise buffer changes contains several Fe^{+++}-positive components. Because of the low resolving power of the forty-five centimeter Dowex 50w-x2 column, the known amino acid hydroxamates and Drosophila hydroxamates were run on the Amino Acid Analyzer. Figure 7 shows the ninhydrin trace of the run of the Drosophila hydroxamates and the Fe^{+++}-positive trace of aliquots of each collected fraction of the same run.

Because of the difficulty in separating the small amounts of Fe^{+++}-positive components from the large amount of citrate buffer in lyophilized fractions from the column run just described, isolation of these components was carried out with a long Dowex 50w-x2 column with a volatile buffer system. Three per cent of each lyophilized sample from the Dowex 50 column was chromatogrammed and the chromatograms dipped in ferric chloride-butanol and ninhydrin solutions. Shown in


1.5
1.0
.6
.5
.4
.3
.2
.1
Absorbance
A
B
50
100
150
200
250
300
350
3.25 buffer
4.25 buffer
5.28 buffer
Milliliters of buffer through column

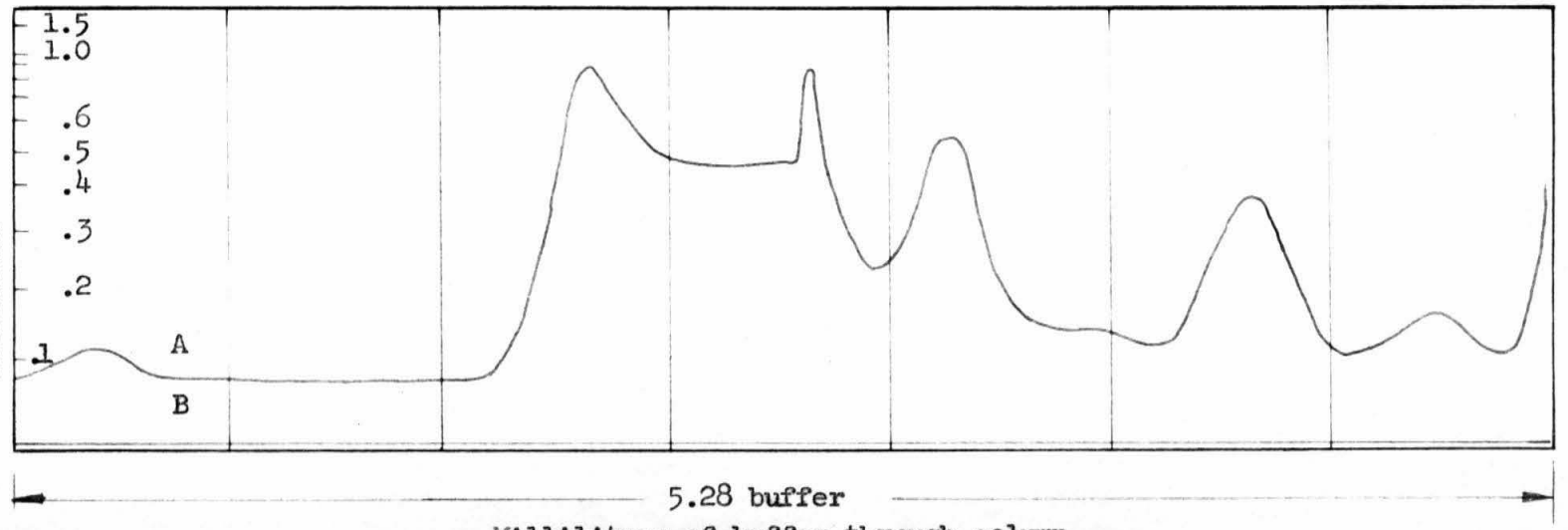

1.5
1.0
.6
.5
.4
.3
.2
.1
Absorbance
A
B
5.28 buffer
Milliliters of buffer through column

Figure 7

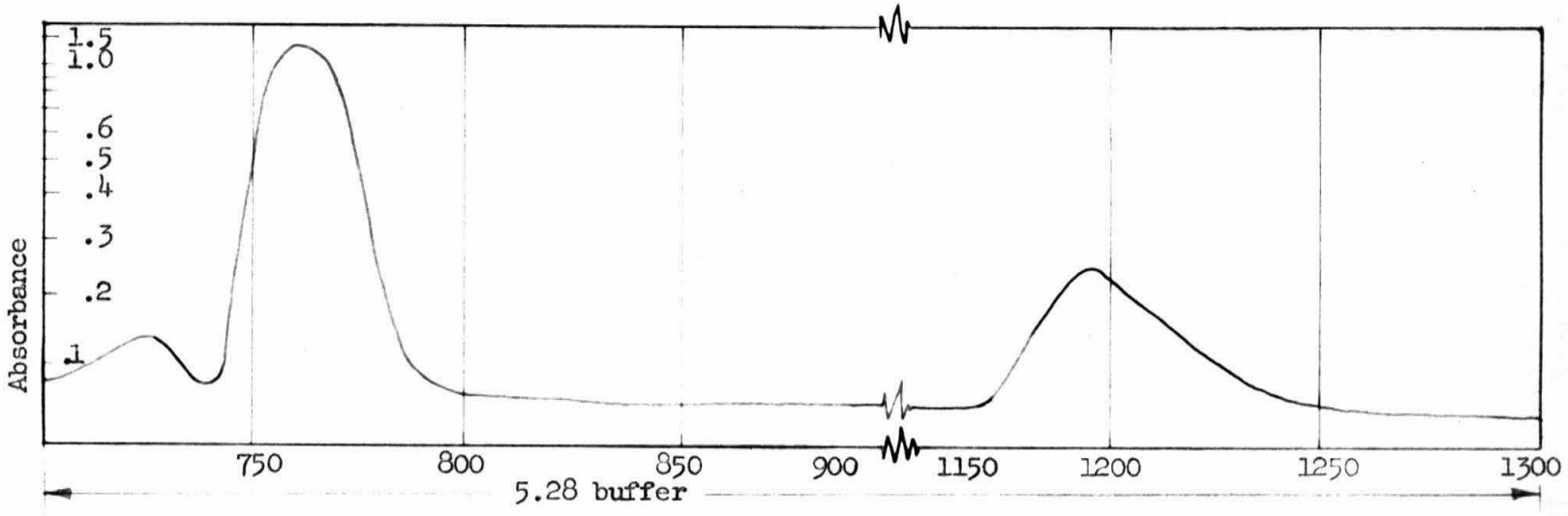


Figure 7 (cont.)

Elution diagram resulting from ion exchange chromatography on Amberlite-120 resin of a pupae homogenate. The sample was prepared by homogenizing ten grams of 122 ± 2 hour Drosophila pupae in twenty milliliters of cold tap water. The homogenate was centrifuged at 8,000 rpm for thirty minutes. Eighteen milliliters of supernatant were recovered and incubated with tyrosine at a final concentration of 2×10^{-5} M and hydroxylamine at a final concentration of approximately 1.0 M. After incubation at 25°C for 2.5 hours, the total reaction mixture was dialyzed against 250 ml of distilled water for twenty-four hours and then lyophilized. The dried sample was dissolved in 2.5 ml of sample diluting buffer, pH 2.20 (citrate buffer, 0.20 N in sodium ion) and applied to the column. The eluting buffers were prepared according to the instruction manual. The buffer changes are indicated in the figure. The temperature was maintained at 52°C throughout the run. The column was 155.5 cm high. Trace A is ninhydrin color at 570 mμ of ten per cent of the sample; the remaining ninety per cent was collected in a fraction collector by means of a stream splitting device. The Fe^{+++}-positive material was determined on aliquots from the collected fractions in the usual manner (trace B). The arrow near 150 ml represents the position of aspartic acid. The scale for the Fe^{+++}-positive material is absorbance and the scale for the ninhydrin color is absorbance x 10.

Figure 8 is a representation of the Fe^{+++}-positive components from the column. The Rf of the compounds is plotted against the tube number and the shaded areas indicate the occurrence of the Fe^{+++}-positive compounds. The details of the column chromatography are given in the legend to Figure 8.

The Fe^{+++}-positive regions of Figure 8 are generally free of ninhydrin-positive materials. This is probably due to the strong ionic character of amino acid and peptides, and they consequently exhibit a very low Rf in the butanol-acetic acid-water solvent. Several of the Fe^{+++}-positive compounds move well away from the base line and can be separated from the ninhydrin-positive compounds by chromatography and elution of the regions of interest. Rechromatography in the propanol-ammonia solvent and another elution produced samples which were hydrolyzed in 6 N HCl for nine hours at 105°C.

To date most of the characterization has been done on the acidic compounds (A, B, C) that were collected in fractions 15 through 20 of Figure 8. Although these compounds can be distinguished from each other by chromatography in butanol-acetic acid-water, they cannot be differentiated by high voltage electrophoresis (5,000 volts for ninety minutes) in eight per cent formic acid. This suggests that they have a similar charge-to-size ratio, but that their partition coefficients between the bound water on the chromatography sheet and the solvent are significantly different.

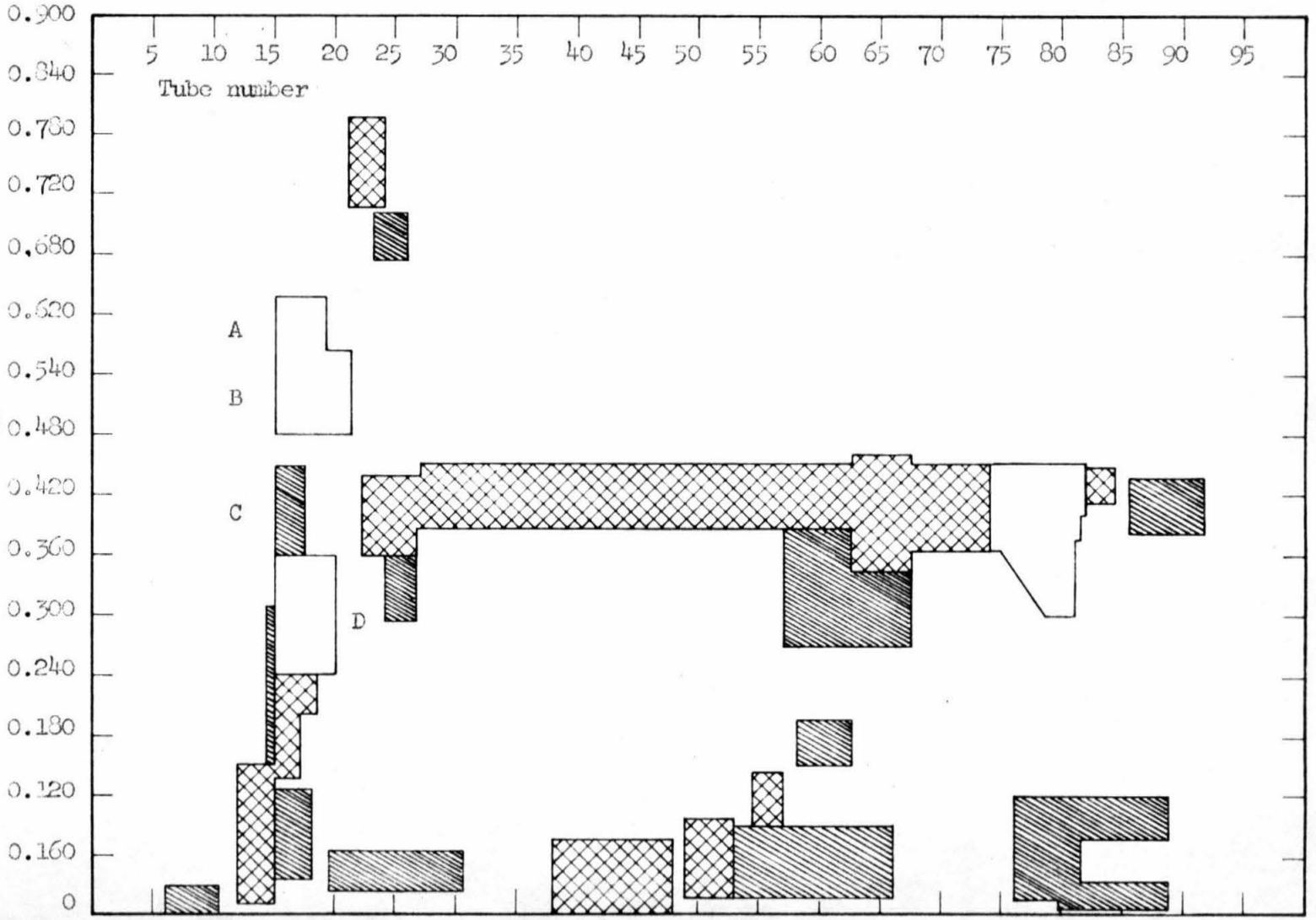
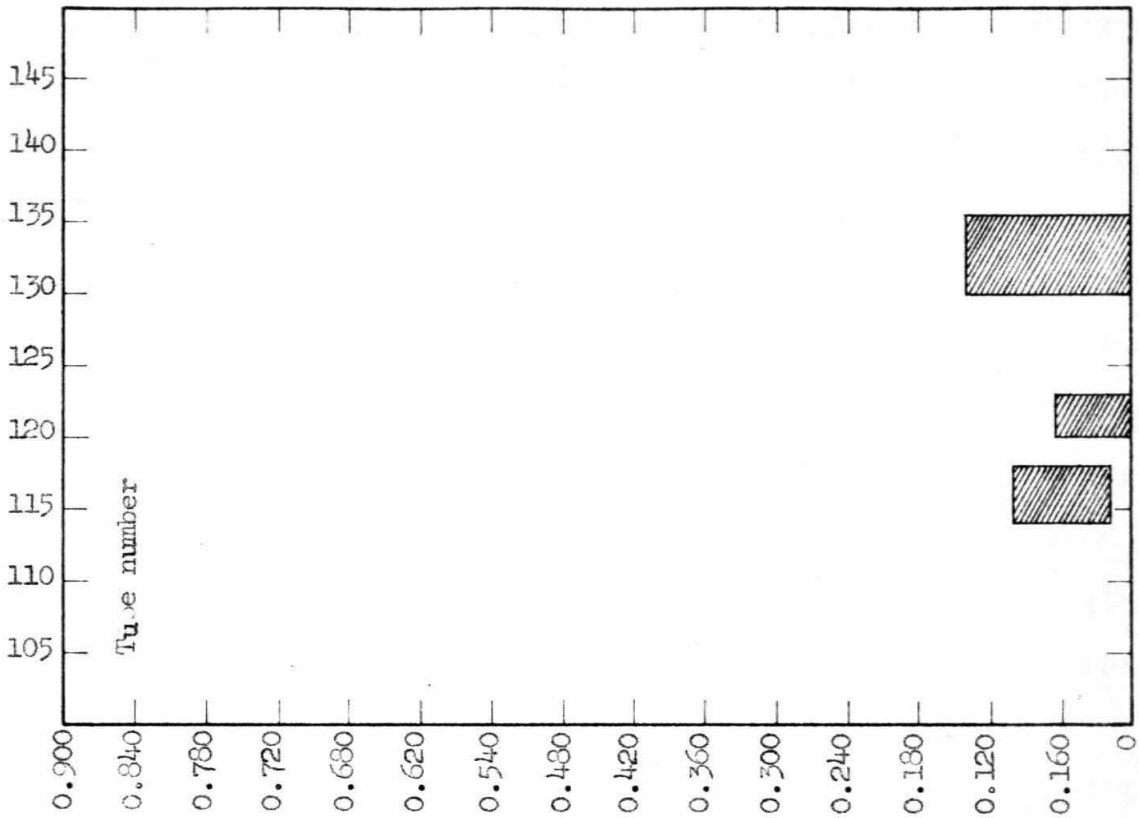

0.900
0.840
0.780
0.720
0.680
0.620
0.540
0.480
0.420
0.360
0.300
0.240
0.180
0.120
0.160
0
Rf
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95
Tube number
A
B
C
D

Figure 6



Chromatographic regions of Fe^{+++}-positive material. 13.7 grams of Drosophila pupae were homogenized, centrifuged 8,000 rpm for thirty minutes, and incubated with hydroxylamine at a final concentration of approximately 1.0 M. After dialysis the lyophilized material was dissolved in 3 ml of 8.8% formic acid and put on a 155 x 1 cm Dowex 50w-x2 column. The resin was in the formate form and had been equilibrated with pyridinium formate buffer, pH 2.36. Elution was begun with this buffer also. The elution schedule was as follows:

Buffer	pH	Prepared by mixing:		Buffer Change
10% pyridine- 8.8% formic acid	2.36	362 ml acid	50 ml pyridine	18
15% pyridine- 13.2% formic acid	2.36	286 ml acid	50 ml pyridine	41
20% pyridine- 17.6% formic acid	2.36	275 ml acid	50 ml pyridine	61
25% pyridine- 22 % formic acid	2.36	245 ml acid	50 ml pyridine	82
25% pyridine- 22 % formic acid	2.61	185 ml acid	50 ml pyridine	100
25% pyridine- 22 % formic acid	2.97	122 ml acid	50 ml pyridine	123
25% pyridine- 22 % formic acid	3.12	83 ml acid	50 ml pyridine	end

The buffer was changed when the indicated tube was under the column. A total of 141 fractions of 5 ml were collected, and the column was kept at room temperature by means of a water bath and pump. Three per cent of each lyophilized sample was chromatogrammed and the chromatograms were dipped in 5% ferric chloride in butanol. The Fe^{+++}-positive regions were marked and transposed to the above diagram. Diagonal lines indicate weakly positive areas, cross-hatched areas are of medium strength and unlined areas are strong Fe^{+++}-positive areas.

Figure 8 (cont.)

The hydrolyzed samples were compared with equal amounts of unhydrolyzed material by chromatography and electrophoresis. There are two major changes produced by the treatment with strong mineral acid: the ability to give a color with ferric ions is destroyed and the response to ninhydrin is now positive. All of the samples which were ninhydrin-negative give more than one ninhydrin-positive component after hydrolysis. Each of the compounds contains much more glutamic acid than any other amino acid, but other amino acids have been tentatively identified by their electrophoretic behavior. Glycine, alanine, and tryptophan as well as one amino acid that belongs to the serine-valine-isoleucine group of this electrophoretic system are present. In addition, component A contains a rapidly migrating, unidentified compound while both B and C contain a slow moving, but different, compound.

The large component in fractions 75-80 has also been subjected to hydrolysis, and, although it is ninhydrin-negative before hydrolysis, it yields at least five amino acids of roughly equal amounts after treatment with mineral acid. Tyrosine, leucine, and arginine are probably present as well as two amino acids that have Rf's of between 0.15 and 0.20 in the propanol-ammonia chromatographic system. The paper blank is negative to ninhydrin treatment before and after hydrolysis so it is felt that the appearance of the ninhydrin-positive material is not an artifact of the methods employed in purification--a common phenomenon if unwashed paper is used for chromatography and elution.

DISCUSSION

Existence of Peptides in Drosophila

Considerable evidence was cited in the introduction which showed that the existence of free peptides in biological materials is a common phenomenon. Additional evidence for this phenomenon is presented in the experimental results of this study. In Figure 7 the ninhydrin trace of an amino acid analyzer run shows large amounts of ninhydrin-positive material from Drosophila pupae which are more acidic than aspartic acid. The only common amino acids which emerge ahead of aspartic acid in this system are methionine sulfoxide and cysteic acid. Since this analysis is of a dialyzed preparation which is protein free, acidic peptides are good candidates for this ninhydrin-positive material which comes off the column in the first 150 ml. Figure 6 also shows such ninhydrin-positive material which emerges before aspartic acid (arrow). Since the samples for Figure 6 were much smaller than that for Figure 7, the multi-component nature of the pre-aspartic acid region is evident.

In Figures 6 and 7 there are other ninhydrin-positive components which are not readily identifiable as amino acids. The known amino acids are marked in Figure 6; they were determined by making a standard calibration run of known amino acids before and after the Drosophila material was run. Since there are many compounds in Drosophila dialyzates that are not

identifiable as amino acids using the amino acid analyzer, it is suggested that these non-protein, ninhydrin-positive substances are peptides. Simmons (5), Simmons and Mitchell (132), and Mitchell and Simmons (133) have done more specific chemical characterizations of such compounds and have concluded that they behave as peptides.

As will be discussed later in greater detail, there are compounds in these Drosophila supernatants that can sustain nucleophilic attack and whose derivatives yield several ninhydrin-positive components upon hydrolysis. This presents the possibility that the peptides which exist in Drosophila homogenates are metabolically active in a special manner.

Hydroxamate Formation

Hydroxylamine reacts with compounds that are capable of sustaining nucleophilic attack; carbonyl-containing compounds are important in this respect for they form hydroxamic acids upon treatment with hydroxylamine. The common species that undergo hydroxaminolysis are esters, acid chlorides, anhydrides, nitriles and imides (134). To result in hydroxamic acid formation the reaction species must have an acyl carbon atom and the reaction must involve an acyl to oxygen bond breaking process. Hydroxamate formation is most certainly a bimolecular reaction of the common $B_{AC}2$ mechanism where B indicates that the substrate rather than the conjugate acid is attacked by the nucleophile, AC indicates that acyl-oxygen

bond breaking occurs, and "2" refers to the molecularity of the reaction (135). The most likely candidates for hydroxaminolysis which exist in biological material are high energy esters and phospho-anhydrides.

Figure 2 shows the plot of hydroxamate formation as estimated by 540 mμ absorption after treatment with ferric ion. Curve A demonstrates that the reaction goes faster at 25°C than at 0°C. The hydroxamate forming system is markedly enhanced by the addition of tyrosine, but there is no satisfactory explanation of this phenomenon at the present time. Mitchell's experiments (130) showing that radioactive tyrosine is rapidly converted to several other components at 0°C are still under investigation. The increase in hydroxamates cannot be accounted for by all the added tyrosine being activated and trapped by hydroxylamine. Approximately three micromoles of tyrosine were added in the experiment described in Figure 2. After two hours of reaction time the difference between curves A and B is about 0.8μmole/ml. For a total volume of fifteen milliliters this represents an increase of 12.0μmole of hydroxamates. Thus there is a fourfold enhancement of hydroxamates compared with what would be expected if the activation of tyrosine was the only source of the increased hydroxamate formation. Although, as judged by the Rf's of the strong Fe^{+++}-positive components from ion exchange chromatography, tyrosine hydroxamate could be present, the behavior of known amino acids on the 40 cm Dowex 50

column suggests that tyrosine hydroxamate does not emerge in the regions of strong Fe^{+++}-positive components. Likewise, there is no explanation for the decrease in the slope of Curve B at the temperature change other than the coincidence of the termination of the tyrosine effect at $0^{\circ}C$ and the raising of the temperature. The slopes of both curves at $25^{\circ}C$ are essentially the same.

Figure 3 demonstrates that the 40,000 rpm supernatant can carry out the reactions necessary for hydroxamate formation and also shows that the preparations vary from day to day. Curves A and C should be quite similar, but they are very different at $0^{\circ}C$. The simplest explanation for the daily variation is that the physiological age of the pupae cannot be controlled as precisely as is necessary and desired.

Figure 4 shows that the fraction sedimenting between 8,000 rpm and 40,000 rpm has a definite effect on hydroxamate formation. This fraction should contain the mitochondria as well as the microsomes since they are generally considered to sediment at about 15,000 rpm. When this sediment is added back by resuspension, the increase in hydroxamates is pronounced (compare curves A and C). The addition of tyrosine further increases the hydroxamate formation in the portion containing the resuspended sediment (Curve D). Tyrosine does not seem to have an enhancing effect on hydroxamate formation in the 40,000 rpm supernatant (Curves A and B). Since the tyrosine effect on hydroxamate formation appears to be involved with the fraction that sediments between

8,000 and 40,000 rpm, one might feel encouraged to investigate this curious effect with respect to the mitochondria and energy generating systems in addition to exploring the possible role of microsomes with respect to tyrosine enhancement of hydroxamate formation.

It is quite clear that Drosophila homogenates can carry out reactions, that form compounds which complex with ferric ion only after the addition of hydroxylamine to the reaction mixture. The obvious candidates for these compounds are hydroxamic acids.

Alkaline Phosphatase

Because of the meager data and the complexity of the system, the significance of the relationships among the alkaline phosphatase activities in Figure 5 is not well understood. It is assumed that the activity is a measure of the concentration of this particular protein in a dynamic system. The decrease of activity is presumably due to proteolytic action by endogenous enzymes; the increase in activity does not necessarily result from de novo synthesis of protein for activation of zymogens is well-known in enzyme chemistry. In any event, some interesting speculations can be made about Figure 5.

The tyrosine and hydroxylamine effects probably are independent phenomena. The addition of tyrosine causes a rise with subsequent decline at both temperatures while the activity of the untreated supernatant increases only after the

temperature change. The behavior of the untreated supernatant probably reflects the fact that some compound is released during the 0° incubation and is not utilized until the temperature is raised. A good candidate for this compound might be tyrosine (130). The action of tyrosine in increasing the phosphatase activity could be the making up of a tyrosine deficiency at this stage of the life cycle. Alternatively, tyrosine or metabolic products might act as an agent for the breaking down or larval protein into small units that could be utilized for the synthesis of adult protein. The tyrosine effect may even be mediated by some derivative such as tyrosine-O-phosphate which is known in Drosophila (135). Mitchell (130) has suggested that dopa phosphate might contain a high energy phosphate group if it is oxidized at the 4 position. Thus the tyrosine effect may not be intimately involved with the protein molecules themselves.

The most attractive explanation for the hydroxylamine effect is the trapping of carboxyl activated intermediates of the general protein synthesis scheme. If hydroxylamine is added, there is an initial rise in activity so hydroxylamine probably does not inactivate the alkaline phosphatase molecules per se. Once the activity starts to drop it is not rescued by the presence of tyrosine or an increase in the temperature. This is consistent with a mechanism that prevents synthesis of new protein while the existing pool is digested by proteolytic enzymes, although there are other plausible explanations.

Although no satisfying statement can be made about the mechanisms of the tyrosine and hydroxylamine effects, there is no doubt that they have curious and striking effects on the alkaline phosphatase activity. These phenomena present interesting possibilities for investigation of cellular control and metabolism.

Effect of Tyrosine and Hydroxylamine on Ninhydrin-positive Compounds

The four ninhydrin traces in Figure 6 show some striking differences. It is felt that these differences are real because of the care that was employed in the preparation of the samples for analysis.

The similarity of the supernatants that have had hydroxylamine added is greater than the similarity to each other of those that are without hydroxylamine. In the acidic region there are several differences between the two hydroxylamine curves; there are notable differences in the region between 65 and 70 ml and between 90 and 120 ml. The difference between the two is particularly striking in the large peak at 110 ml. Beyond the acidic region there are a few small differences between the hydroxylamine curves; this is evident with the small unidentified peaks near methionine, between leucine and isoleucine, and between leucine and tyrosine. In the hydroxylamine-treated samples, phenylalanine and lysine are the only variations of known amino acids; the amounts of the other known amino acids are strikingly similar.

Thus, tyrosine in the presence of hydroxylamine appears to have a greater effect on the very acidic and other minor components than it does on the known amino acids and the unidentified neutral components.

The curves without hydroxylamine can only be compared with each other in the acidic and part of the neutral regions due to the loss of the larger part of run I. As is readily seen, these curves are more similar to each other than to the hydroxylamine curves, but they are quite different between themselves. The presence of tyrosine causes an increase of those substances which emerge in the first 150 ml, and then the tyrosine-containing trace (III) is the same or slightly lower than trace I with the one exception at 145 ml.

In comparing the two sets of curves (I and III with II and IV) we can see that the total amount of material in the first 180 ml of effluent is much less if hydroxylamine is present. This could be due to the prevention of the formation of these acidic substances or to their transformation to other compounds. With the data at hand neither the formation of hydroxamic acids nor any other means of decreasing the acidic material with hydroxylamine can be strongly suggested. Some refinements that could be introduced into this type of experiment are (1) starting the run at a lower pH to spread out the many components in the acidic region (2) finding a means to sharpen the peaks in the basic region

(3) exploring the region beyond arginine and (4) isolating specific compounds and studying their specific behavior in relation to the overall pattern.

This work with the amino acid analyzer clearly emphasized that both the addition of tyrosine and hydroxylamine have rather drastic effects on the metabolism of ninhydrin-positive compounds in Drosophila supernatants. A good example of this is that added tyrosine in the absence of hydroxylamine does not show up greatly in the ninhydrin pattern (trace III), however, in the presence of hydroxylamine, the tyrosine peak is obvious whether tyrosine has been added or not.

The Nature of Fe^{+++}-positive Materials

The nature of the compounds formed by Drosophila supernatants under the influence of hydroxylamine has not been thoroughly investigated. Some information is available however; they are not amino acid hydroxamates for they do not exhibit the same behavior on ion-exchange columns as known hydroxamates. A number of them are acidic and come off Dowex 50 and Amberlite 120 columns in the first 100 ml of effluent. Further purification of these column peaks by chromatography on paper produces samples which are free of ninhydrin-positive material. Hydrolysis of these compounds releases several amino acids as identified by chromatography and electrophoresis. Those compounds which emerge from cation

exchanger columns in the acidic region show a predominance of glutamic acid which is interesting in view of the fact that polyglutamic acid has been found in Drosophila larvae (5,130). All of these compounds which have been freed of ninhydrin-positive materials by chromatography have produced five to seven amino acids after hydrolysis. Hydrolysis destroys the ability of the samples to give a purple color with ferric ion. The information that is available about these compounds at the present time is certainly consistent with a peptidyl hydroxamate model.

The information gained thus far about these compounds certainly suggests that a further detailed study is in order. The facts that their formation is absolutely dependent on hydroxylamine and that a large variety of them are present after hydroxylamine treatment of Drosophila supernatants make them interesting subjects for further investigations. Since none of them has been isolated in pure form, it is not possible to make conclusive statement about their nature.

Peptides and Protein Synthesis

The introduction to this thesis contains a large amount of evidence that peptides and peptide derivatives are naturally-occurring classes of compounds in biological materials; there is also a considerable amount of evidence that these classes of compounds are active in the normal metabolic pathways of organisms. Although peptide intermediates in protein

synthesis have generally not been considered for several years, there are a few investigations which have recently been concerned with this possibility.

Since Drosophila pupae are quasi-closed systems, they appear to be a reasonable place to study peptide metabolism; thermodynamics would favor peptide intermediates for at least one high energy bond would be conserved per intact peptide bond that is incorporated into adult protein. Selection pressure in evolution should favor such a mechanism in highly specialized forms such as insect pupae.

This work has shown that a trapping reagent, hydroxylamine, has a definite effect on the activity of an enzyme as well as a significant alteration of the distribution of small ninhydrin-positive compounds in Drosophila homogenates. From the small amount of work designed at revealing the mechanism of the trapping reagent, it appears that peptides are likely to be among the compounds influenced by such a nucleophile.

It is felt that the reasons that this work varies considerably from the bulk of the investigations on protein synthesis are because of the approach taken in this work and because of the specialization of Drosophila pupae. Since it is felt that peptides are well worth considering as intermediates in protein synthesis in Drosophila pupae, it may be worthwhile to make some conjectures about the mechanism of such a process. The possible mechanism that immediately comes to mind is the activation and transfer of peptides to sRNA by the means that

amino acids of the current protein biosynthetic scheme are activated and transferred (see Figure 1). If the C-terminal amino acid of a peptide is transferred to the messenger RNA-covered surface of the ribosome, it is plausible to suggest that the rest of a short peptide could find its way onto the ribosomal surface if the sequence of the peptide is the correct one. This raises some questions about the indiscriminate production of peptides by endogenous proteolytic action. There has been no good explanation for the specific proteolytic action of some enzymes such as chymotrypsin in biological materials; there is a possibility that specific proteolytic action and the functions of peptides are not unrelated topics.

Another possible way of putting peptides on the ribosome is for the N-terminal and the C-terminal amino acids of a peptide to be complementary to adjacent sites on the messenger RNA surface (130). This mechanism would allow for a small region of messenger RNA to specify a relatively long portion of a polypeptide chain. If the adaptor hypothesis is universal, or even correct, it does not particularly rule out peptide intermediates. The peptide could reach over adjacent sRNA sites to the next charged sRNA molecule.

Several bits of evidence have been presented that are not inconsistent with peptides as intermediates in protein synthesis. This work, in a familiar pattern, has raised more questions than it has answered; of course, much further investigation is needed to delineate the real nature of the problem. It is felt that the results presented here make a good case for further investigation in this area.

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