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PUTRESCINE AND POLYAMINES:
RELATION TO GROWTH AND
DEVELOPMENT IN DROSOPHILA
MELANOGASTER

ARNOLD SILVA DION

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RELATION TO GROWTH AND DEVELOPMENT IN
DROSOPHILA MELANOGASTER

by

ARNOLD S. DION

B.S. University of New Hampshire, 1964

M.S. University of New Hampshire, 1966

A THESIS

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This thesis has been examined and approved.

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January 10, 1969

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ABSTRACT

PUTRESCINE AND POLYAMINES:
RELATION TO GROWTH AND DEVELOPMENT IN
DROSOPHILA MELANOGASTER

by

ARNOLD S. DION

Although many investigations concerned with the levels of putrescine ($\text{H}_2\text{N}(\text{CH}_2)_4\text{NH}_2$), spermidine ($\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$), and spermine ($\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$) in various tissues and organisms have appeared, no study has been conducted on the levels of these compounds throughout the entire life cycle of a complex organism. This investigation was initiated in an attempt to correlate putrescine and polyamine levels as a function of growth and development in Drosophila melanogaster.

A quantitative fluorescent technique has been modified to permit the analysis of putrescine, spermidine, and spermine. The application of this technique to the developmental stages of D. melanogaster has revealed high levels of spermidine concomitant with the initiation of each stage, i.e. embryonic, larval, pupal, and adult. In addition stage maturation is characterized by decreasing spermidine levels and a spermidine/putrescine ratio which approaches unity. A possible antagonism

between spermidine and putrescine, with regard to RNA synthesis, is suggested. Measurements of certain biochemical growth parameters, i.e. RNA, DNA, and protein, indicate a relationship between high biosynthetic activity and the level of spermidine.

The administration of C¹⁴-putrescine and C¹⁴-methionine to sterile larvae aged 1.5 hours indicated only a small conversion to spermidine at a time when the spermidine level is high on a non-axenic medium. Similar results were obtained with larvae aged 104 hours. In addition the administration of C¹⁴-spermidine to the older larvae resulted in the rapid production of labeled putrescine, which, in turn, was rapidly metabolized. These data indicate that D. melanogaster derives spermidine mainly from the diet and/or the biosynthesis of spermidine is dependent upon a nutritionally complete diet. Furthermore, it is suggested that stringent homeostatic controls exist over spermidine levels.

Pupal stages, maintained at 0° C, exhibited rapidly reduced levels of spermidine, probably as a result of acetylation. In contrast late third instar larvae, subjected to temperature shock, exhibited increased spermidine levels. The oral administration of molting hormone, ecdysone, effected both an increase and a decrease in the larval level of spermidine.

Methods have also been developed for the mass isolation of the sex peptide of D. melanogaster, leading to partial characterization.

LITERATURE REVIEW

THE DIAMINES AND POLYAMINES

Literature concerned with the diamines and polyamines has been extensively reviewed by Tabor and Tabor (161) for the period extending to September, 1964. A literature review extending to November, 1966 has been compiled by Dion (47); therefore, the following review is intended to develop an historical perspective, and will concentrate on literature of interest published since November, 1966. The early literature dealt almost exclusively with the occurrence of diamines and polyamines in various organisms and tissues, and with the elucidation of their chemical structures. It was not until Herbst and Snell (80,81) observed that putrescine was a growth factor for Hemophilus parainfluenzae that evidence for a physiological role for diamines and polyamines was obtained.

1. Occurrence

Herbst et al. (82) have demonstrated that, in general, gram negative bacteria contain high diamine and polyamine levels, while gram-positive bacteria have low levels. The gram-negative bacterium, Escherichia coli, was reported to contain 15 μ moles of putrescine, 1.5 μ moles of spermidine, 0.3 μ moles of monoacetyl putrescine, 0.1 μ moles of monoacetyl spermidine, and 0.5 μ moles of a glutathione-like conjugate of spermidine per gram wet weight, when grown on minimal media (52,54). Recent experiments by Tabor (153) indicate that these

estimates are probably incorrect, since cold-harvested E. coli cells rapidly acetylate spermidine. Whether concomitant modifications occur to the diamines and other polyamines was not mentioned. Therefore, diamine and polyamine levels reported in cold-harvested organisms should be regarded with caution.

Variations in culture conditions have been shown to produce dramatic changes in diamine and polyamine levels in microorganisms. E. coli cells grown in an alkaline medium contained more spermidine than putrescine (158). The addition of triethanolamine or spermidine increased intracellular levels of putrescine and spermidine and their acetylated derivatives. The addition of spermine resulted in the replacement of putrescine and spermidine with spermine and its acetylated derivatives. The alteration of intracellular diamine and polyamine levels has also been noted in lactic acid bacteria (77), and in a Pseudomonas sp. (94).

Significant changes in diamine and polyamine levels have also been effected by the addition of antibiotics. Raina and Cohen (132) demonstrated an enhancement in the biosynthesis and accumulation of spermidine in cells of E. coli strain 15TAU treated with chloramphenicol; streptomycin moderately increased the level of spermidine, but caused putrescine leakage into the medium. A loss of putrescine and an increase in spermidine was also noted with the addition of levorphanol (146).

The nitrogenous compounds reported by Hershey (84) to be

injected with the viral DNA now appear to have been putrescine and spermidine. High concentrations of putrescine and spermidine have been reported in the T-even bacteriophages of E. coli B (7,8) and bacteriophage 3 of E. coli 518 (92). However, no diamines or polyamines were detected in bacteriophage T3, Salmonella typhimurium bacteriophage P22, tobacco mosaic virus, cucumber virus, or polio virus (8). That the polyamines can substitute for the inorganic cation requirement for phage multiplication within the host, has been demonstrated for T4rII mutants in E. coli K12 lysogenic for the lambda prophage (33). Shalitin (141) has suggested that putrescine effects selective gene transcription of early messenger RNA in phage T4.

The occurrence of putrescine has been noted in Datura stramonium, Atropa belladonna, orange juice, barley seedlings, chinese cabbage, mushrooms, and various other plants (see review by Tabor and Tabor, 161). It has also been reported (44,135) that putrescine accumulates in potassium-deficient plants and in Vicia faba leaves, when these plants are maintained in a medium high in NaCl. The occurrence of putrescine, spermidine, and spermine has been noted by Dion (47) in V. faba roots; however, spermine was only detected in the meristematic region. In contrast Bagni et al. (24) reported no detectable levels of spermidine or spermine in roots and anthers of wheat; however, all other portions of the wheat plant contained these

polyamines. Appreciable levels of the polyamines have also been noted in embryos of various cereals (114). Polyamines have been reported to be constituents of some plant alkaloids (161) and putrescine is a probable precursor of nicotine (105). Putrescine, but not polyamines, was found to occur in coconut milk (23). It is interesting to note that spermine (28), spermidine, putrescine, and cadaverine (23) stimulate the cellular proliferation of Helianthus tuberosus.

Poisonous secretions of the bird spider, Pamphobeteus tetracanthus, contain spermidine and 1,3-diaminopropane in amide linkage with p-hydroxyphenylpyruvic acid (60). Ackermann (2,3) noted that spermine, spermidine, and putrescine occur in pupae of the silkworm, Bombyx mori; Dion and Herbst (49) also reported the presence of these compounds in Drosophila melanogaster. Biosynthetic pathways leading to arcaine (1,4-diamidinoputrescine) and to hirudonine (1,7-diamidinosperspermidine) in the leech, Hirudo medicinalis, have been elucidated (10,136).

Polyamines have long been known as ubiquitous components of mammalian organs (161). Rosenthal and Tabor (137) and Raina (129) established the presence of spermine and spermidine in the formed elements of human blood. In addition Shimizu et al. (142) found higher levels of spermine in blood containing nucleated erythrocytes. Polyamine analyses of human brain tissue have revealed striking differences between

6

gray and white matter. Gray matter contained high levels of spermine and lower levels of spermidine; the converse was realized with respect to white matter. Low levels of putrescine and its monoacetylated derivative have also been detected in human brain (124).

The relationship between diamine and polyamine levels and rapid cell proliferation in carcinoma, organ regeneration, and growth processes has been of particular interest. Kosaki and his collaborators have reported the presence of spermidine in a phospholipid, malignolipin, exclusively in malignant tissue; however, these reports have been refuted by a number of authors (see Tabor and Tabor, 161). Free polyamines have been found in mouse mammary carcinoma, sarcoma, hepatoma (137), and Ehrlich ascites cells (15).

Spermidine and spermine levels have been investigated by Raina (130) in the developing chick embryo. Two polyamine concentration maxima were observed, and these correlated well with RNA maxima. A similar correlation between spermidine and RNA levels in regenerating rat liver was observed by Dykstra and Herbst (56); however, the level of spermine remained relatively unchanged. The relationship between growth hormone and spermidine levels was investigated by Kostyo (97). Hypophysectomy effected a reduction in spermidine concentration in rat hepatic tissue; however, normal levels were reestablished within 24 hours after bovine growth hormone administration; spermine levels remained unchanged.

2. Quantitative Methods

Recent developments have greatly increased the sensitivity of quantitative methods for diamines and polyamines. After preliminary extractions, diamines and polyamines have been separated by paper chromatography (53,79,82), paper electrophoresis (59,130), and ion-exchange chromatography (155,166). Ninhydrin (59) or amido black (130) have served as location reagents for diamines and polyamines separated by paper methods; 2,4-dinitrofluorobenzene derivatives of these compounds have been utilized after ion-exchange separations.

Recent methods of analysis have included the synthesis of fluorescent derivatives, colored complexes, and/or separation by thin layer chromatography. Unemoto et al. (166) have described the ion-exchange separation of spermine and spermidine, and oxidation of these compounds by beef plasma amine oxidase in the presence of resorcinol, yielding fluorescent products. The condensation product of spermidine and o-phthaldehyde can also be determined fluorometrically (57).

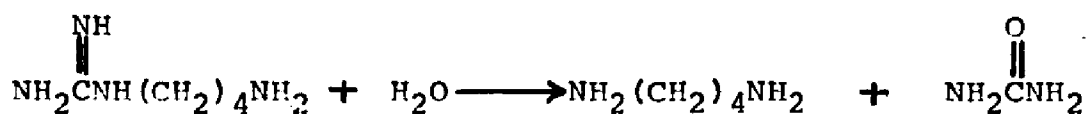
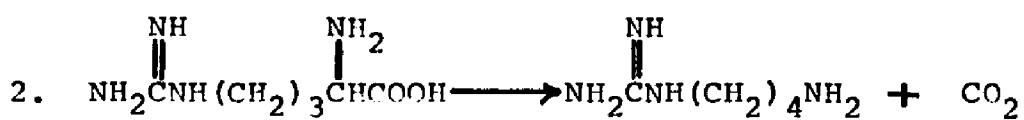
Colored complexes, in addition to those previously mentioned, have also been utilized in quantitative methods. These include the reactions of Δ^1 -pyrroline, the oxidation product of spermidine by the action of dried Serratia marcescens cells, with o-aminobenzaldehyde (18); the reaction of oxidized spermine and spermidine with N-methyl-2-benzothiazolone hydrazone hydrochloride (22), and the reaction of spermine with platonic iodide (11).

A complex method involving ion-exchange chromatography and thin layer chromatography on cellulose and silica gel layers has been reported (86). A more suitable method has been communicated by Hammond and Herbst (78) and includes the separation of diamines and polyamines on cellulose thin layers, reaction with ninhydrin, and elution and quantitation of the colored complexes. A highly sensitive method for the direct quantitation of the dansyl (1-dimethylamino-naphthalene-5-sulfonic acid chloride) amide derivatives of spermine and spermidine, separated by silica gel G thin layer chromatography, has been devised by Seiler and Wiechmann (140). Modifications in the latter procedure by Dion et al. (48) also allow the quantitative determination of putrescine. The mass spectrophotometric analysis of these dansyl derivatives, eluted from silica gel layers, has been proposed by Creveling et al. (45) for positive identification. Finally, a quantitative immunochemical method involving polyamine haptens, which would obviate extraction and separation of diamines and polyamines from tissue, is being developed (128).

3. Biosynthesis and Degradation

Evidence for biosynthetic pathways leading to spermidine and spermine was first obtained with E. coli and Aspergillus nidulans (59,156,157). The decarboxylation of ornithine yielded putrescine, which was found to be incorporated into spermidine and spermine. The aminopropyl moiety of spermidine

Putrescine biosynthesis has recently been extensively investigated in E. coli by Morris and collaborators (110,111, 112). Two major biosynthetic pathways have been shown to be active in the formation of putrescine:

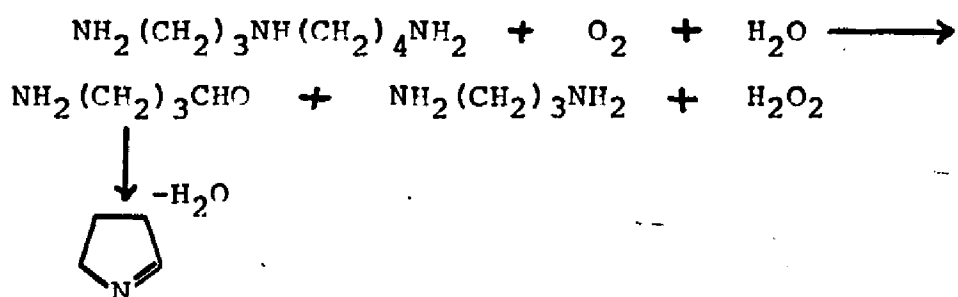


The first pathway yields putrescine through the decarboxylation of ornithine. In the second pathway agmatine is formed by the decarboxylation of arginine; agmatine is then deamidated to putrescine and urea.

That putrescine and/or methionine are precursors for polyamines has been demonstrated for rat prostate (121,156), liver, kidney, skeletal muscle, and brain (131), and the developing chick embryo (130). In addition the rate of formation of these compounds in rat liver has been investigated as a function of age (144), regeneration (56,87), starvation and growth hormone administration (87,144).

The oxidative degradation of various diamines by animal diamine oxidase has been extensively investigated and reviewed

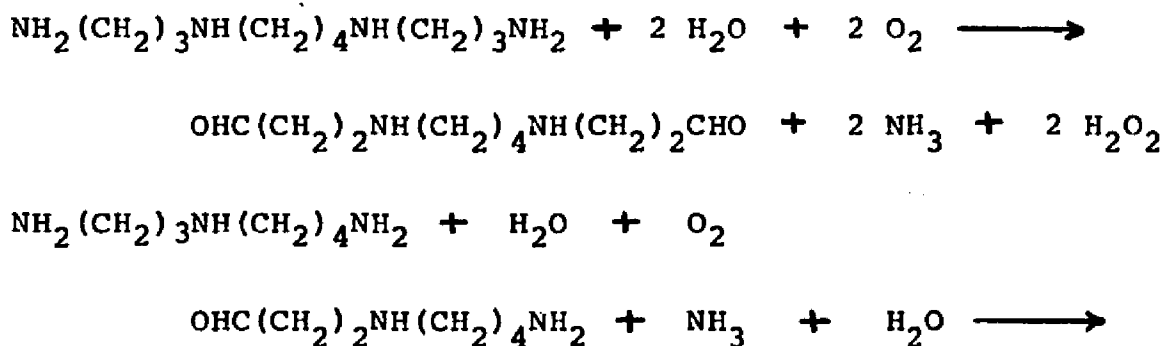
by Zeller (170). Michaels and Kim (109) assayed a large number of microorganisms for putrescine degradation, and Yamada et al. (168,169) investigated putrescine oxidase derived from Micrococcus rubens. The purification of an enzyme from S. marcescens which degrades spermidine and bis-(3-aminopropyl)-amine, by the following reaction, has been reported by Bachrach (12,13):



Δ^1 -pyrroline

The oxidation of spermidine in a Pseudomonas sp. (119) yields putrescine and 3-amino-propionaldehyde.

A soluble plasma amine oxidase has been purified (160,165) and shown to deaminate spermine and spermidine oxidatively by the following reaction (162,165):



The physiological activity of oxidized polyamines,

especially oxidized spermine, has recently been extensively investigated by Bachrach and collaborators. Oxidized spermine effects the inactivation of plant and bacterial viruses (17, 21), transforming DNA (125), gram-negative and gram-positive bacteria (19), and Ehrlich ascites cells (14). Investigations concerning the mode of action of oxidized spermine have revealed immediate inhibition of RNA synthesis in E. coli; protein synthesis was inhibited soon thereafter (20). From these and additional data Bachrach et al. (16) have postulated that oxidized spermine induces covalent interstrand cross-linking of DNA.

4. Diamine and Polyamine-Cell Organelle Interactions

Acid-base interactions have been suggested as an explanation for the stabilizing effect of the polyamines, spermine and spermidine, on membrane systems. Spermine has been demonstrated to bind to spheroplasts, phospholipids, and cell wall constituents (134,152). Mager (104) and Tabor (149,152) demonstrated lysis inhibition of spheroplasts and protoplasts by spermine and spermidine. A related phenomenon may explain the reduction in swelling in isolated mitochondria by spermine, spermidine, putrescine, and cadaverine (83,149). Recently, Stevens (148) reported that a number of spermine analogues, viz. $\text{NH}_2(\text{CH}_2)_n\text{NH}(\text{CH}_2)_x\text{NH}(\text{CH}_2)_n\text{NH}_2$ where n is 3 and x is 2-6, commensurately stabilized E. coli protoplasts (sic). The stabilization of microsomal membranes by spermine and spermi-

dine is believed to result in the conversion of estradiol to water-soluble metabolites (90).

Cohen and Lichtenstein (42) observed that approximately 15% of the total diamines and polyamines of an E. coli extract was associated with ribosomes, and that they were non-exchangeable. Similar findings were reported by Kim (94) for putrescine in a Pseudomonas sp.; however, Kim (94) and Siekevitz and Palade (143) demonstrated uptake of spermine by ribosomes of E. coli, a Pseudomonas sp. and pig pancreas, respectively. Tabor and Kellogg (154) found that ribosomal amines in E. coli were exchangeable, and that this exchange was dependent on isolation conditions. Redistribution of diamines and polyamines in various cell fractions derived from rat liver has been examined by Dykstra (55), Raina and Teleranta (133), and Khawja and Stevens (93).

The maintenance of bacterial 70S ribosomes by the polyamines is well documented (42,43). Although magnesium was also capable of stabilizing these particles, maximal stabilization occurred when both polyamine and magnesium were present in the suspending medium. That magnesium cannot fully replace polyamine in the above systems, has also been suggested by Pestka (126).

The following relationship between ribosomal structure and ionic milieu has been proposed by Watson (167) and confirmed by Norton et al. (117):

demonstrated to be enhanced by the presence of polyamines, possibly through the abovementioned interactions. Brewer and Rusch (32) noted that spermine enhanced DNA polymerase activity in isolated nuclei of Physarum polycephalum; however, this stimulation was observed only in the presence of exogenous DNA. In contrast, spermine inhibition of DNA synthesis was observed in isolated mitochondria (31). Polyamine-induced stimulation of RNA synthesis has been observed in in vitro bacterial systems (66,98), isolated rat liver nuclei (103), isolated Dipteran salivary glands (49), and in the chick embryo (113). A number of hypotheses have been advanced to account for this stimulation and includes:

1. Polyamine reversal of product inhibition via the dissociation of the enzyme-bound RNA (66).
2. Polyamine modification of the RNA product so as to render it unable to bind to the template site (98,1).
3. Stabilization of native DNA structure (1).
4. Polyamine modification of the secondary structure of defective single-stranded regions of DNA, resulting in an increase in enzyme molecules available for RNA synthesis (1).

Translational processes also appear to be stimulated by polyamines as indicated by the enhancement of amino acid incorporation into rat liver microsomes and ribosomes (85), and in E. coli ribosomes (50). In addition diamines and polyamines markedly increased phenylalanine incorporation in the presence of polyuridylic acid in a system containing S. typhimurium

ribosomes (108). A possible mechanism by which polyamines interact at the translational level may be indicated by data demonstrating that spermidine enhances the binding of tyrosyl transfer RNA to ribosomes with uridylyl-(3',5')-adenylyl-uridine in a yeast system (163). Cocucci and Bagni (40) have claimed that spermidine and putrescine induce the activation of protein synthesis in Helianthus tuberosus. Spermine has been shown to inhibit protein synthesis in Walker 256 cells (71), Staphylococcus aureus (67), and in mouse ascites cells (118). In all cases excessive stabilization of anticodon-codon binding was concluded to be the mechanism of inhibition.

THE SEX PEPTIDE OF DROSOPHILA MELANOGASTER

In attempting to develop paper chromatographic method as an adjunct to taxonomic studies, Fox (63) discovered an extra ninhydrin-positive spot in males of D. melanogaster, which he termed "sex peptide". Chen (36) failed to confirm Fox's results; however, this lack of confirmatory evidence was shown by Fox et al. (64) to be attributable to the chromatographic solvents employed. In addition the latter authors reported the presence of sex peptide in males of 16 genotypes, and its absence in females of 34 genotypes. That the sex peptide is the product of sex determining genes, was demonstrated by the presence of sex peptide in genetic females transformed into sterile males by the third chromosome recessive mutant transformer.

Acid hydrolysis of the sex peptide (65), eluted from paper chromatograms, released ten ninhydrin-positive products. Paper chromatographic analyses of these free residues and their dinitrophenyl derivatives revealed the presence of: aspartic and glutamic acids, serine, glycine, α -alanine, leucine, valine, methionine, ethanolamine, and one unknown.

The physiological significance of the sex peptide was investigated by Chen and Diem (37). Isolation and paper chromatographic analyses of the abdominal organs of male flies demonstrated that the sex peptide was confined solely to the paragonia, which are approximated by the prostate glands

of higher organisms. Since the paragonia arise from larval imaginal discs (30), it was assumed that transplantation of the latter into males would increase the sex peptide content of these males. Similarly, it was assumed that transplantation into females would effect the appearance of sex peptide. Both of these assumptions were verified by experimentation. Chen and Diem also investigated the growth of the paragonial organs and its relationship to sex peptide content. Sex peptide content was low in males less than 5 days old; however, there was a gradual increase in peptide in males 5-9 days of age.

A number of physiological functions have been postulated for the paragonial organs (123). According to Nonidez (116) the paragonial secretion of Drosophila furnishes the liquid portion of the ejaculate. Garcia-Bellido (69) has postulated that this secretion is responsible for sperm motility and acts as a stimulus for the opening of the seminal vesicle. The relationship between paragonial secretion and female fertility was initially investigated by Gottschewski (72), and later expanded by Leahy. Leahy and Lowe (101) observed a dramatic increase in oviposition in virgin females injected with saline or methanol extracts of male paragonia. That the enhancement of oviposition is non-specific for Dipterans, was demonstrated by Leahy (100). Heterologous transplants among the Diptera, i.e. Aedes aegypti, Culex pipiens, and D. melanogaster, resulted in significant increases in oviposition;

however, when Dipteran paragonial glands were transplanted into non-Dipterans, viz. Tenebrio molitor and Tenebrio obscurus, negative results were obtained. It should be noted that the sex peptide has been alternatively termed the "paragonia secretion" and the "male factor" by Chen and Diem, and Leahy, respectively; however, based on chromatographic behavior these terms appear to be synonymous.

METHODS AND MATERIALS

I. Drosophila melanogaster

D. melanogaster cultures, originally maintained on a standard yeast medium, were obtained from Professor Edythe Richardson, Department of Biology, University of New Hampshire. The original culture was multichromosomal for sepia eye and miniature wing; however, these phenotypes are rarely encountered in the present culture, which has been maintained for almost 5 years by repeated sib-matings. These cultures were maintained on the following medium (120) at $25 \pm 0.5^\circ \text{C}$, modified by the addition of propionic acid, with a 12:12 hour light/dark cycle:

Solution A:

sucrose	500 g.
$\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ (NaK Tartrate)	50 g.
$(\text{NH}_4)_2\text{SO}_4$	12 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3 g.
CaCl_2	1.5 g.
H_2O for 3000 ml.	

Solution B:

Bacto-Agar (Difco)	135 g.
$\text{C}_4\text{H}_6\text{O}_6$ (Tartaric Acid)	30 g.
KH_2PO_4	4 g.
Propionic acid	2.7 ml.
H_2O for 3000 ml.	

The final solution was prepared by adding equal amounts of solutions A and B and dissolving by heating. A piece of paper toweling was added to each culture to provide a dry surface for pupation. It should be noted that the above medium will not alone suffice to maintain D. melanogaster cultures, since these organisms have fastidious nutritional requirements (138); however, it has long been observed that the transfer of adults to stock new cultures always results in the growth of microflora on the surface of the medium. This microorganism must therefore serve as the primary source of nutrition for these cultures, which are probably monoxenic.

Since published methods for axenic culture were cumbersome to employ, the following method was devised. 200-300 pairs of adult flies were transferred without anesthesia to a 250 ml. Erlenmeyer flask which was stoppered with a cork stopper into which was inserted a short-stem powder funnel. This apparatus was inverted over a Petri dish containing a layer of the above medium. Egg-laying was allowed to proceed for a period of 3 hours. The eggs were then swept from the surface with a "camel's hair" brush and deposited in Ephrussi-Beadle medium (7.5 g. NaCl, 0.35 g. KCl, and 0.21 g. CaCl₂ per liter of aqueous solution). The collected eggs were then filtered through a Millipore apparatus (Millipore Filter Corp., Bedford, Mass.) equipped with a type HA membrane filter. The eggs were dechorionated in the presence of commercial Clorox diluted with an equal volume

of water for 5 minutes. This solution was filtered and the eggs were washed with Ephrussi-Beadle medium. The eggs were then washed with 70% ethanol containing 0.05% HgCl_2 . Finally, the eggs were washed with Ephrussi-Beadle medium. All of the above steps were performed within a Millipore apparatus which was fitted with a rubber stopper into which was inserted a piece of glass tubing filled with cotton to filter incoming air. The filter containing the dechorionated and sterilized eggs was transferred under a germicidal ultraviolet lamp to the following sterile medium, which is a modification of Sang's medium C (138):

The following were dissolved in 50 ml. distilled H_2O :

$\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$	0.9300 g.
$(\text{NH}_4)_2\text{SO}_4$	0.1992 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.0498 g.
CaCl_2	0.0250 g.
$\text{C}_4\text{H}_6\text{O}_6$	0.5000 g.
KH_2PO_4	0.0664 g.

To the above solution was added 0.4 g. Yeast nucleic acid (Calbiochem, Los Angeles, Calif.), which was allowed to dissolve. This was followed by the addition of 5.5 g. vitamin free casein (Nutritional Biochemicals Corp., Cleveland, Ohio) and 3 g. Bacto-Agar (Difco Laboratories, Detroit, Michigan). After the addition of 3.0 ml. of lipid suspension (stock solution consisted of 0.3 g. cholesterol and 4.0 g. lecithin dissolved in 30 ml. warm ethanol and 30 ml. distilled H_2O ;

the ethanol was removed by autoclaving), the volume was adjusted to 70 ml. and sterilized. Sucrose solution (9.3 g./27 ml. H₂O) was autoclaved separately and added to the above. Finally 3 ml. of a vitamin solution, containing the following constituents, were added through a Millipore Swinnex 13 filter unit:

Thiamine	0.0002	g.
riboflavin	0.0010	g.
nicotinic acid	0.0012	g.
Ca pantothenate	0.0016	g.
pyridoxine	0.00025	g.
biotin	0.000016	g.
folic acid	0.0003	g.

All of the above vitamins were obtained from Calbiochem, Los Angeles, Calif.

2. ANALYSES

DIAMINES AND POLYAMINES

Each designated stage was collected, washed with Ephrussi-Beadle medium, dried on filter paper at room temperature and weighed. This tissue was then transferred to a Kontes ground-glass Duall microhomogenizer, 0.4 ml. 0.2 N perchloric acid (PCA) was added, and the tissue was homogenized with a fitted

ground-glass pestle. The homogenate was then centrifuged for 10 minutes in a Fisher microcentrifuge, and 0.3 ml. of the supernatant was transferred to a screw-cap tube; the PCA-insoluble precipitate was saved for protein analysis. To the above aliquot of the supernatant was added 0.4 ml. dansyl chloride (10 mg./ml. acetone; Pierce Chemical Co., Rockford, Ill.), and 50 mg. $\text{Na}_2\text{CO}_3 \cdot 10 \text{H}_2\text{O}$. Dansylation was allowed to proceed for 16 hours at room temperature in the dark. Excess dansyl chloride was destroyed by reaction with 0.1 ml. proline solution (10.0mg./ml. H_2O) for 30 minutes. The dansylated products were then extracted by adding 0.5 ml. benzene and vigorous manual shaking. The benzene layer was removed, transferred to a small screw-cap vial, and immediately frozen at -20°C .

The separation and quantitation of putrescine and the polyamines were effected by the method of Seiler and Wiechmann (14) with modifications. Thin layer plates were prepared by vigorously shaking 30 g. Silica Gel G (nach Stahl, Merck AG, Darmstadt, Germany) and 60 ml. distilled H_2O , and applying the slurry to precleaned glass plates with a Brinkmann-Desaga variable thickness applicator (Brinkmann Instruments, Westbury, N.Y.) set at 200 microns. The resulting plates were air-dried overnight, and activated at 105°C for 1 hour just prior to sample application. Samples and standards were applied with a Hamilton Microliter Syringe approximately 2 cm. from the bottom of the plate. For the determination of putrescine

and spermidine the plates were developed in ethyl acetate/cyclohexane (3:4, v/v); spermine and spermidine determinations were effected after development in ethyl acetate/cyclohexane (1:1, v/v) and ethyl acetate/cyclohexane/triethylamine (50:50:1) in the same dimension.

After development the plates were immediately sprayed with triethanolamine/isopropanol (1:4, v/v) so that approximately 2 g. of triethanolamine was deposited on each plate. The sprayed plates were dried in vacuo in a vacuum oven (National Appliance Co., Portland, Oregon) at room temperature for 16 hours over silica gel dessicant. After equilibration in air for 1 hour, the plates were ready for fluorometric observation.

Direct quantitation of fluorescence intensity was obtained with a Turner Model 111 Fluorometer (G.K. Turner Associates, Palo Alto, Calif.). The light source employed was a Long Wave Source (Turner Catalog #110-850). Activation at 365 m μ was effected with primary filter 7-37 (Turner Catalog #110-834). Filter #2A-12 served as the secondary filter, and allowed the transmission of wavelengths longer than 512 m μ . Fluorescence intensities were recorded on a Rustrak Miniaturized Automatic Chart Recorder.

Since gaussian curves were obtained, the following procedure was used to determine peak areas. Net peak height was obtained by subtracting the base height from the total

peak height. A straight line was drawn through the midpoint of the net peak height parallel to the base line. The width of the line subtended by the curve was measured. The peak area was determined as the product of one-half the peak height and the width. Standard curves were prepared by plotting moles of standard amine solutions versus peak area.

PROTEIN

Protein was determined by the method of Kuno and Kihara (99). 0.9 ml. 0.2 M MgCl_2 was added to the PCA-insoluble residue from the preceding analysis, and the residue was rehomogenized. 0.1 ml. was then transferred to a screw-cap test tube, and 9.9 ml. 0.2 M MgCl_2 were added. 1.0 ml. was then passed through a membrane filter (Schleicher and Schuell Co., Type B-6, 24 mm.) under reduced pressure. The filter was washed with 3.0 ml. 0.2 M MgCl_2 , and the protein was stained with 2.0 ml. Elphor amidoschwarz (2 $\mu\text{g}/\text{ml}$. of acetic acid/methanol/ H_2O , 1:5:4) obtained from Bender and Hobein, Munich, Germany. Excess stain was removed with 5.0 ml. 1% acetic acid. The color was then eluted with 3.5 ml. 0.01 M NaOH, and the eluate was read at 620 $\text{m}\mu$. A standard graph was prepared from bovine serum albumen (0-50 μg) treated as indicated above.

NUCLEIC ACIDS

The indicated stages were washed in Ephrussi-Beadle medium, dried on filter paper, transferred to a Kontes microhomogenizer, and homogenized in 0.5 ml. cold 95% ethanol. After 15 minutes the homogenate was centrifuged and the supernatant was discarded. 0.5 ml. methanol-chloroform (2:1, v/w) was added, the residue was resuspended, left for 15 minutes at 4° C, and centrifuged. 1.0 ml. ice cold 0.3 N PCA was added to the lipid-free residue, rehomogenized, and centrifuged. This procedure was repeated one more time. For third instar larvae and pupae, the PCA suspension was dialysed against 0.01 M triethanolamine (pH 7.0) at 45° C for 2 hours to remove uric acid. The residue was then extracted 2X with 1.0 ml. 1 N PCA at 80° C for 20 minutes each time.

For the determination of RNA, duplicate 0.4 ml. aliquots were made up to the final volume of 1 ml. by the addition of distilled H₂O. 1.0 ml. of orcinol reagent (concentrated HCl containing 0.1% FeCl₃·6H₂O and 0.1% orcinol recrystallized 3X from benzene) was then added to each sample, and the samples were heated for 20 minutes in a boiling water bath. The samples were cooled and read at 670 mμ. Standards of yeast RNA were simultaneously run at 0-100 μg/ml.

DNA was determined by the diphenylamine reaction as

modified by Burton (34). Duplicate 0.4 ml. samples were added to 0.6 ml. of 1 N PCA. Two volumes of diphenylamine reagent (1.5 g. diphenyl-amine, 100 ml. glacial acetic acid, 1.5 ml. concentrated H_2SO_4 , and 0.5 ml. water solution of acetaldehyde, (16 mg./ml. were then added. After an incubation of 17 hours at 26-27° C, the optical density was read at 600 m μ . Standards were simultaneously run at concentrations of 0-100 μ g/ml. of salmon sperm DNA.

3. THIN LAYER TECHNIQUES

Cellulose thin layer plates were prepared by adding 75 ml. distilled H_2O to 30 g. Whatman Cellulose Powder (CC-41), slurring by manual shaking, and applying the slurry to 5 20 X 20 cm. glass plates with a variable thickness spreader set at 250 microns. These plates were air-dried overnight, and were ready for use. Silica Gel plates were prepared by slurring 30 g. Silica Gel G, nach Stahl (E. Merck AG, Darmstadt, Germany) with 60 ml. distilled H_2O , and applying the slurry as above at a thickness of 200 microns. The plates were air-dried overnight and employed with either no further treatment (unactivated) or activated by heating at 110° C for 1 hour just prior to use.

Thin layer chromatographic (TLC) separations were effected by applying the sample approximately 2 cm. from the bottom of the plate and developing in the indicated solvent system. For two dimensional separations the sample was

applied to one corner of the plate, 2 cm. from each edge, developed, dried, rotated 90°, and developed a second time.

For thin layer electrophoresis (TLE) the site of sample application was dependent upon the charge and electrophoretic mobility of the compound(s) of interest. Since cellulose usually contains ninhydrin-positive interfering substances, the plates were developed in 1% acetic acid by conventional TLC methods, dried, the solvent front was scraped off, and the sample was applied. The layer was wetted by spraying the plate with buffer, and the plate was transferred to a Gelman electrophoresis chamber and power source (Gelman Instrument Co., Ann Arbor, Mich.). Strips of Whatman 3MM paper (4 X 20 cm), wetted with the same buffer, served as wicks.

The following sensitive method was devised for thin layer radioautography (TLR). Clean 20 X 20 cm. glass plates were subbed by dipping in an aqueous solution containing 0.5% gelatine and 0.1% chrome alum, and air-drying. The subbed plates were then layered (200 μ) with NTB-2 liquid emulsion previously liquified at 40° C for 1 hour. For the localization of radioactive compounds, an "emulsion plate" was pressed against the layer of a conventional TLC plate, and the plates were taped along their edges to prevent movement. The plates were stored in a light-proof container, and allowed to expose at 4° C. At the end of the exposure period, the emulsion plate was developed in Dektol developer diluted 1:1, stopped in

acetic acid (30 sec.), fixed for 5 minutes, and washed with H_2O for 20 minutes.

Preparative TLC and TLE procedures employed layers of 500 μ thickness; all subsequent steps were as indicated above.

Primary and secondary aliphatic amines were located by spraying with 360 mg. ninhydrin (Pierce Chemical Co., Rockford, Ill.) in 35 ml. absolute ethanol and 0.45 ml. collidine (2,4,6-trimethylpyridine). Certain phenols and imidazoles were located by spraying with Pauly reagent, which consisted of 10 ml. 0.9% sulphanilic acid in 0.12 M HCl, 1 ml. 4.5% $NaNO_2$, and 11 ml. 10% Na_2CO_3 . Phosphate-containing compounds were reacted with finely powdered ammonium molybdate (1 g./8 ml. H_2O), concentrated HCl, 12 N PCA, and acetone (8:3:3:86 by volume). Background color of the molybdatespray was eliminated by placing the sprayed plate in a covered jar containing a beaker of concentrated NH_4OH .

4. MASS ISOLATION OF SEX PEPTIDE

2-5 g. of frozen ($-20^\circ C$) Drosophila melanogaster males were homogenized in 80% methanol (1/8, w/v) in a Kontes homogenizing tube with a Teflon pestle. The homogenate was then centrifuged at 2000 rpm in an International Centrifuge (Head #812) for 10 minutes at $0-2^\circ C$. The supernatant was removed and saved, and the pellet was rehomogenized in 80%

methanol, centrifuged, and the supernatant was removed. This procedure was repeated once more, and the pooled supernatants were reduced in volume to approximately 5 ml. in vacuo. The resulting sample was then applied to a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.27 X 168 cm.) equilibrated with distilled H₂O, and having a void volume of 86.5 ml. The sample was eluted with distilled H₂O, and the fractions (1.5-2.5 ml.) were monitored at 220 and 250 mμ. After the appropriate fractions, containing sex peptide, were pooled and lyophilized, the sample was subjected to preparative cellulose TLC and developed in n-butanol/acetic acid/ H₂O (4:1:1, v/v). In this solvent system the sex peptide had an R_f of 0.049; two ninhydrin-positive contaminants appeared at R_f values of 0.00 and 0.137. The cellulose layer containing sex peptide was scraped from the plate and the peptide was eluted with 80% methanol. After taking the peptide to dryness in vacuo, it was stored at -20° C.

Amino Acid Analysis

An aqueous solution of sex peptide was transferred to a test tube, lyophilized to dryness, and 1.0 ml. triple glass-distilled constant boiling HCl (6 N) was added. After evacuation the tube was sealed, and the peptide was hydrolysed at 107° C for 24 hours. The hydrolyzate was taken to dryness in vacuo over NaOH pellets and H₂SO₄. The residue was resuspended in distilled H₂O and lyophilized to remove residual HCl. Finally,

the residue was resuspended in 0.2 M sodium citrate buffer (pH 2.2). Analyses were performed with a Beckman 120 C Amino Acid Analyser (Spinco Division, Palo Alto, Calif.) according to the method of Spackman et al. (147), modified by the use of Custom Spherical Resins for accelerated analyses.

Amino-terminal Analysis

A small aliquot of peptide was transferred to a small test tube, lyophilized to dryness, and the residue was resuspended in 15 μ l 0.2 M NaHCO_3 (73). 15 μ l of dansyl chloride (2.5 mg/ml. acetone) were added and dansylation was allowed to proceed at ambient temperature for 2-3 hours in the dark. The mixture was then lyophilized to dryness, and 50 μ l 6 N HCl were added to the dansylated product. Hydrolysis was allowed to proceed for 18 hours at 105° C. After removal of HCl in vacuo over NaOH pellets and H_2SO_4 , the residue was resuspended in acetone and plated on an activated silica gel G TLC plate. The sample was first subjected to electrophoresis (73) for 2 hours at 350 V. in pyridine/acetic acid/ H_2O (10:20:2500) buffer at pH 4.4, followed by chromatography (76) in benzene/pyridine/acetic acid (80:20:5). The fluorescent products obtained were compared to standards prepared according to the method of Gray and Hartley (74). 6.5 μ moles of the desired standard, dissolved in 1 ml. 0.1 M NaHCO_3 , was reacted with 1 ml. dansyl chloride (6 mg/ml. acetone) overnight at room temperature in the dark. After dansylation 8 ml. acetone were added, the precipitated NaHCO_3 was removed by centrifugation, and the supernatant was plated directly.

RESULTS AND DISCUSSION

1. THE QUANTITATION OF PUTRESCINE AND POLYAMINES AS DANSYL DERIVATIVES

The earlier method described by Seiler and Wiechmann (140) for the quantitation of the dansyl derivatives of the polyamines was of limited usefulness, since it did not allow for the estimation of putrescine, a polyamine precursor and the major amine of many organisms, e.g. gram-negative bacteria. However, since only small amounts of tissue were available in some life stages of D. melanogaster, the adaptation of this ultrasensitive method was essential.

It was soon found that the unknown fluorescent compound in the application of the above method with the solvent system ethyl acetate/cyclohexane (50:50, v/v), was putrescine; however, its proximity to dansyl ammonia prohibited its quantitation. The resolution of dansyl putrescine and ammonia was first attempted by increasing the solvent migration distance by attaching a filter paper wick to the upper end of the TLC plate. Although this method increased the resolution, the sensitivity was decreased because of diffusion. Modification of the solvent system was next attempted, and ethyl acetate/cyclohexane in a proportion of 3:4 (v/v) was found to effect good resolution. The possible interference of other dansyl derivatives was also investigated,

and the R_f values of a number of derivatives are listed in Table 1. In addition it should be noted that dansyl derivatives of the amino acids and various other carbonyl-containing compounds have little or no mobility in the following solvent systems.

Table 1. R_f values of various dansylated derivatives separated by the method of Seiler and Wiechmann (140) and the present method.

Derivative:	$R_f(1):$	$R_f(2):$
ammonia	0.752	0.423
putrescine	0.748	0.322
spermidine	0.551	0.196
spermine	0.453	0.123
1,3-propanediamine	0.703	0.294
cadaverine	0.772	0.344
monoacetylated spermidine A*	0.026	0.012
monoacetylated spermidine B*	0.166	0.094
monoacetylated putrescine	0.026	0.013
adrenalin	0.478	0.210
noradrenalin	0.594	0.148

(1) ethyl acetate/cyclohexane (50:50, v/v) followed by acetate/cyclohexane/triethylamine (50:50:1, v/v).

(2) ethyl acetate/cyclohexane (3:4, v/v).

A*: $\text{CH}_3\text{CONH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$

B*: $\text{NH}_2(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NHCOCH}_3$

Table 1 reveals the possible interference of 1,3-propanediamine and cadaverine with putrescine, and adrenalin and noradrenaline with spermine and spermidine, respectively; however, most tissues contain only minor quantities of these compounds as compared to putrescine and the polyamines. Also, the differences in R_f values are sufficient to distinguish them from the compounds of interest. Typical fluorescence scans, obtained as described in Methods and Materials, are given in Figures 1 and 2. Usually a blue fluorescing compound interferes with the estimation of spermine in the present method; therefore, the use of the Seiler and Wiechmann method is recommended for the quantitation of this compound.

Figure 3 illustrates that a linear relationship exists between area and mole concentration. This relationship is highly reproducible; however, the straight line generated by the standard values generally does not pass through the origin. The latter is the result of a small amount of streaking in the direction of solvent migration, which is also the direction for fluorescence scanning. The error introduced by streaking can easily be corrected by applying the straight line equation: $y = mz + c$. Solving for the slope m , i.e. area/moles, and taking the reciprocal, the following working formula is obtained:

Figure 1. Fluorescence scan of a standard mixture containing 5.0×10^{-10} moles of each component. From left to right the dansylated derivatives of spermine, spermidine, and putrescine.

Figure 2. Fluorescence scan of a dansylated extract obtained from newly emerged adult flies. From left to right blue fluorescing compound, spermine, spermidine, and putrescine.

FIG. 1

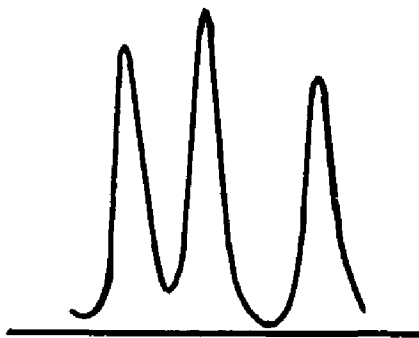
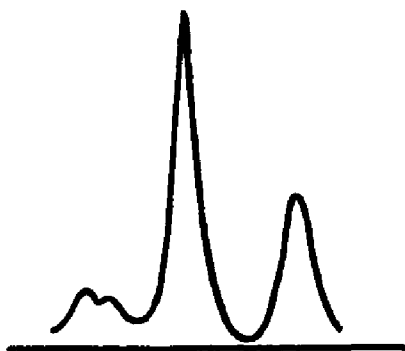


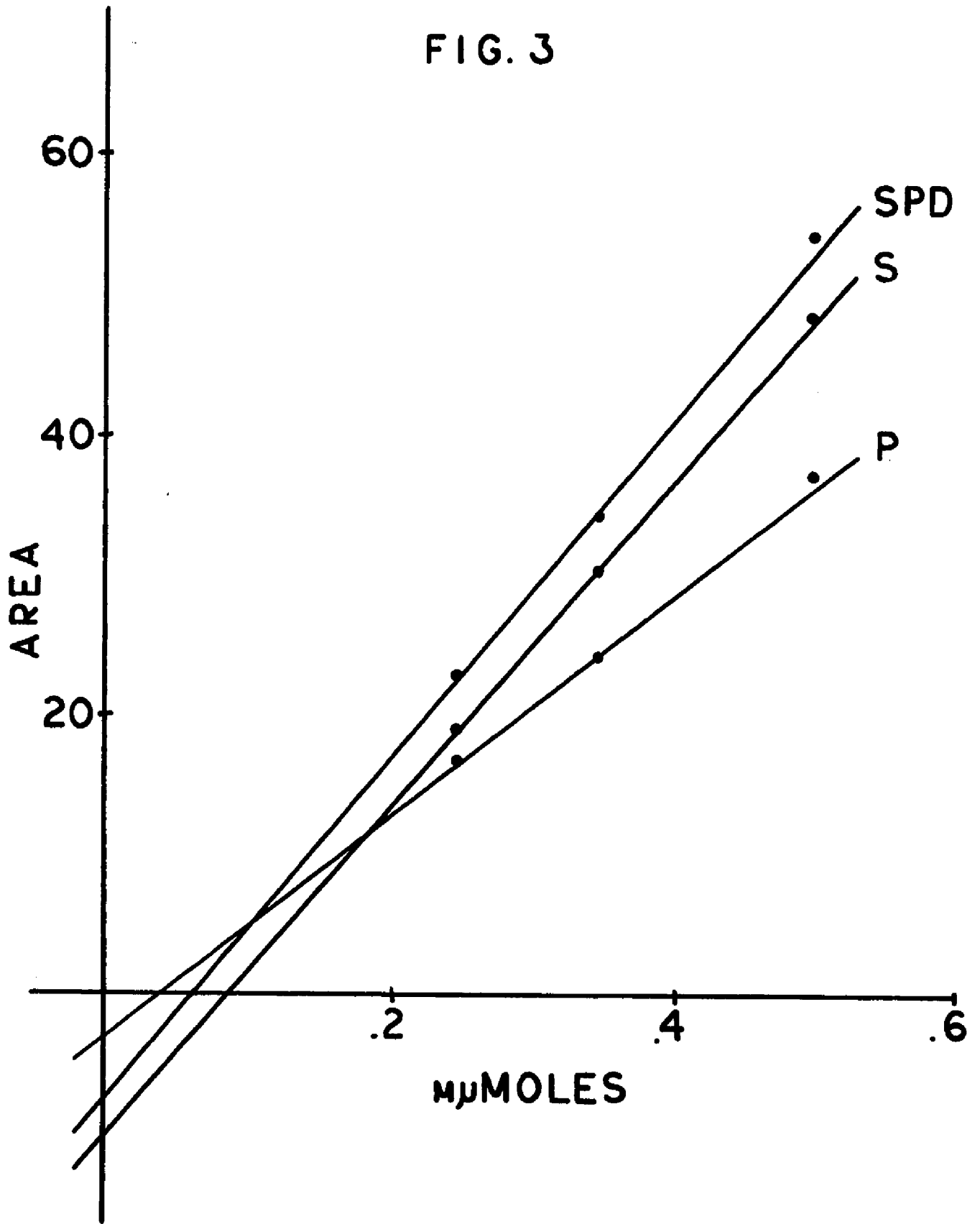
FIG. 2



moles = $x/y-c$ (area), where the area is equivalent to $\frac{1}{2}$ height X width of the sample curve and $x/y-c$ is a constant for a given line.

Figure 3. Standard curve obtained by spotting 5, 7, and 10 μ l aliquots, corresponding to $2.5, 3.5,$ and 5.0×10^{-10} moles of putrescine (P), spermidine (SD), and spermine (S). Areas were obtained as indicated, and are expressed in arbitrary units.

FIG. 3



2. PUTRESCINE AND POLYAMINE LEVELS IN THE DEVELOPMENT
OF DROSOPHILA MELANOGASTER

This is the first attempt to investigate putrescine and polyamine levels in an organism during almost its entire life cycle. The present stock of D. melanogaster is characterized by an embryonic developmental time of approximately 21 hours after oviposition at 25° C. For convenience the time of hatching will be denoted as 0; all designated times are $\pm 1.0-1.5$ hours. White puparium formation and pupation occur at 114 and 126 hours, respectively. The pupal period lasts approximately 85 hours, resulting in the eclosion of the imago.

Figure 4 illustrates putrescine and polyamine levels on a wet weight basis in the developing egg; spermine was present only in trace amounts. The highest levels of putrescine and spermidine were found in eggs incubated for 1.5 hours (preblastula), decreasing rapidly to minimum values at 3.0 hours (blastula). Beginning at 5 hours (gastrula) there was a gradual increase in putrescine and spermidine contents. Interestingly, the levels of putrescine and spermidine in unfertilized eggs (4.0 hours) were observed to be 6.507 and 0.542 μ moles, respectively, per gram wet weight. In contrast putrescine and polyamine levels in the unincubated chick embryo have been reported to be nil (35). It is conceivable

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that fertilization in D. melanogaster triggers the conversion of putrescine to spermidine, and that putrescine may play a role as antagonist to the physiological function(s) of spermidine. The latter point will be discussed more fully in the following text. The contents of putrescine and spermidine per egg are given in Figure 5, and followed a similar pattern to those obtained on a wet weight basis. The latter is the result of near wet weight constancy during embryonic development in D. melanogaster.

Polyamine levels in the chick embryo (21 day development time) have been investigated by Raina (130). In this system two polyamine maxima were observed at 2 and 15 days when calculated on a wet weight basis. On a per embryo basis a constant increase in the polyamines was observed up to the sixteenth day. Although some of the differences in polyamine concentration between these two systems may be rationalized on the basis of wet weight changes in the chick embryo, polyamine content changes within individual embryos still remain, and probably reflect basic differences in nucleic acid metabolism.

Figure 6 illustrates putrescine and spermidine levels in larval, pupal, and adult stages on a wet weight basis. Spermidine maxima occur in the early larval stage (3 hours), in the prepupal-early pupal stage (122 hours), and in virgin adult females aged 291 hours. Two putrescine maxima are

observed in larvae aged 49 hours, and in virgin adult males aged 291 hours.

In Figure 7 are given data concerning putrescine and spermidine levels on a per organism basis in the above designated stages. The most noteworthy changes in spermidine maxima have occurred in the larval stage, i.e. the early larval (3 hours) maximum has been replaced by a maximum in larvae aged 77 hours. These data are indicative of concomitant spermidine and wet weight increases in larval stage; however, since the wet weight increases at a faster rate, the maximum at 77 hours is masked in Figure 6. Spermine levels relative to Figures 6 and 7 are given in the appendix.

The larval levels of putrescine and spermidine are striking, since this stage fed on a microorganism which has vastly different putrescine and polyamine contents relative to this stock of D. melanogaster. The levels of putrescine and polyamine in this microorganism have been observed to be 0.3805, 0.1111, and 0.0556 μ moles putrescine, spermidine, and spermine, respectively, per gram wet weight. A spermidine/putrescine ratio of 0.29, which is not observed in any stage, indicates that the intestinal tract probably contributes little to the total putrescine and polyamine content of the organism. In addition the larval stage must be capable of modifying ingested putrescine by elimination, destruction (e.g. oxidation), modification (e.g. acetylation), and/or

conversion to the polyamines.

Spermidine/putrescine ratios as a function of development are presented in Figure 8. High ratios are observed in eggs incubated for 3-5 hours, in larvae aged 3-29 hours, 77 hours, late larvae, in pre- and early pupae, and in adult females. Interestingly, the ratio of spermidine/putrescine in adult males is close to unity throughout the adult stage. The relationship of high spermidine/putrescine ratios and RNA synthesis will be the subject of the next section.

Figure 4. Putrescine and spermidine concentrations (μ moles) per gram wet weight in fertilized eggs of D. melanogaster incubated for the designated times at 25° C. Solid line: spermidine; dashed line: putrescine.

Figure 5. Putrescine and spermidine concentrations (μ moles) per fertilized D. melanogaster egg incubated for the designated times at 25° C. Solid line: spermidine; dashed line: putrescine.

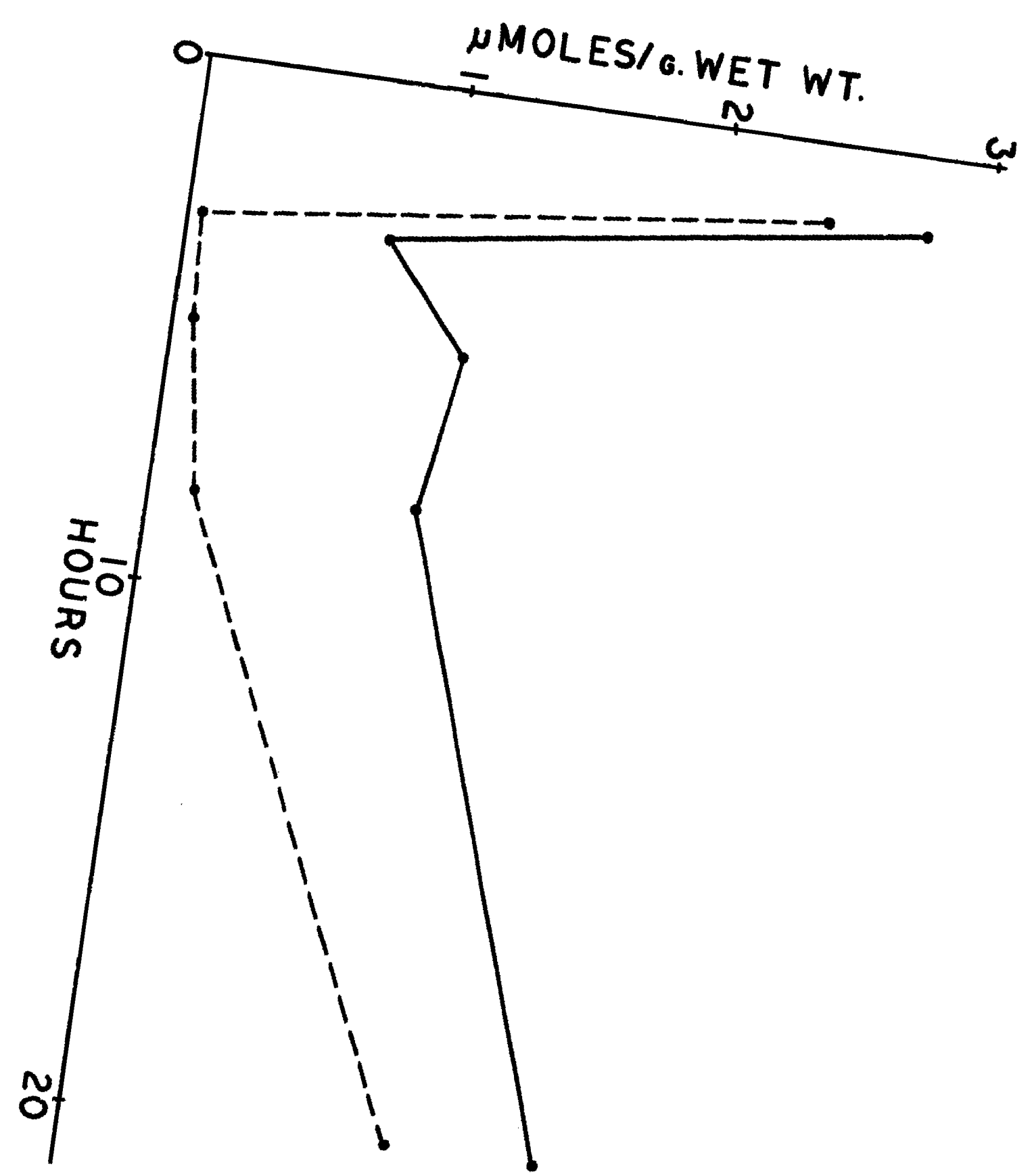


FIG. 4

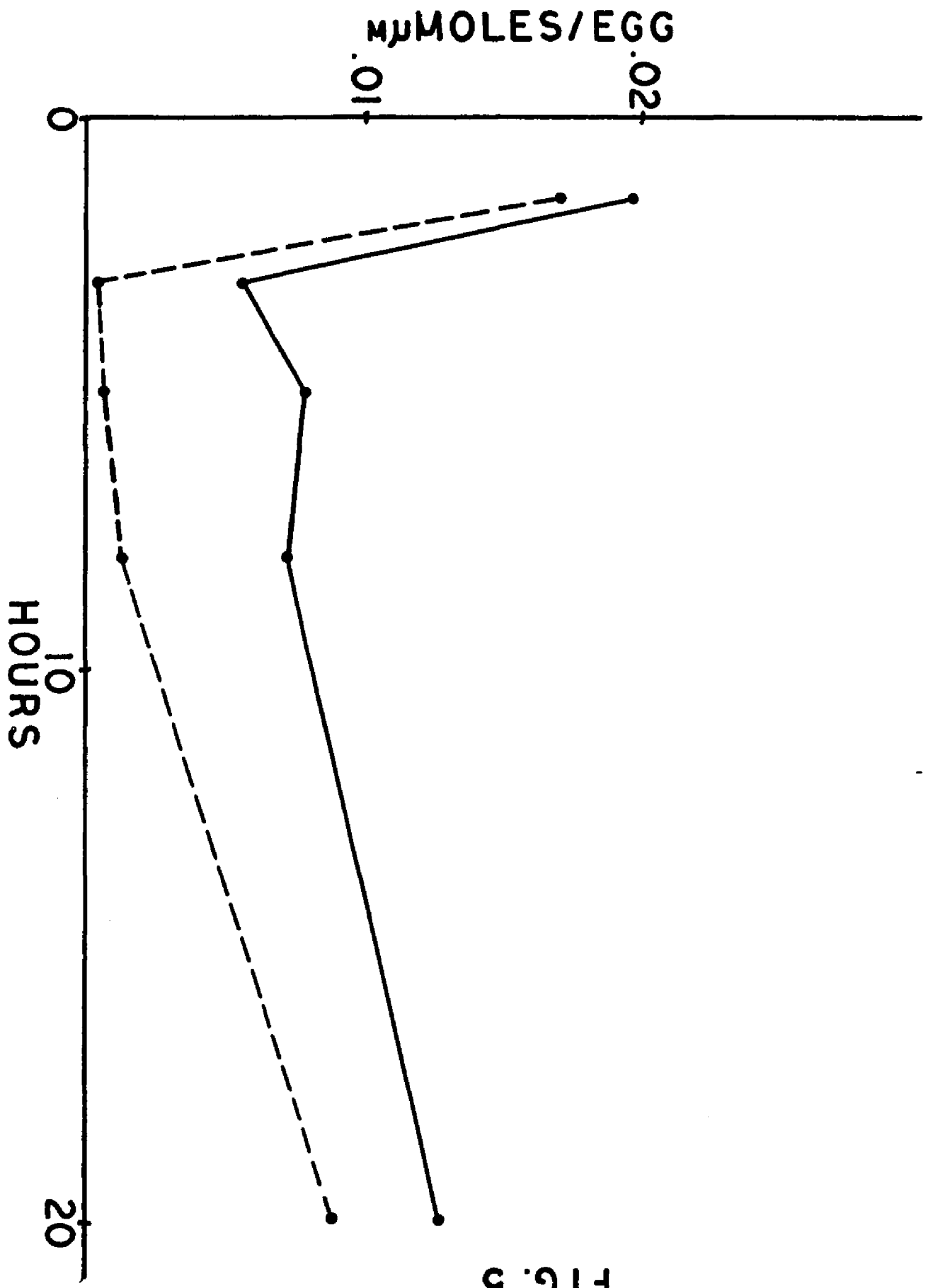


FIG. 5

Figure 6. Putrescine and spermidine concentrations (μ moles) per gram wet weight in larval, pupal, and adult stages of D. melanogaster at 25° C. Solid line: spermidine; dashed line: putrescine.

Figure 7. Putrescine and spermidine concentrations (μ moles) per individual organism at 25° C. Solid line: spermidine; dashed line: putrescine.

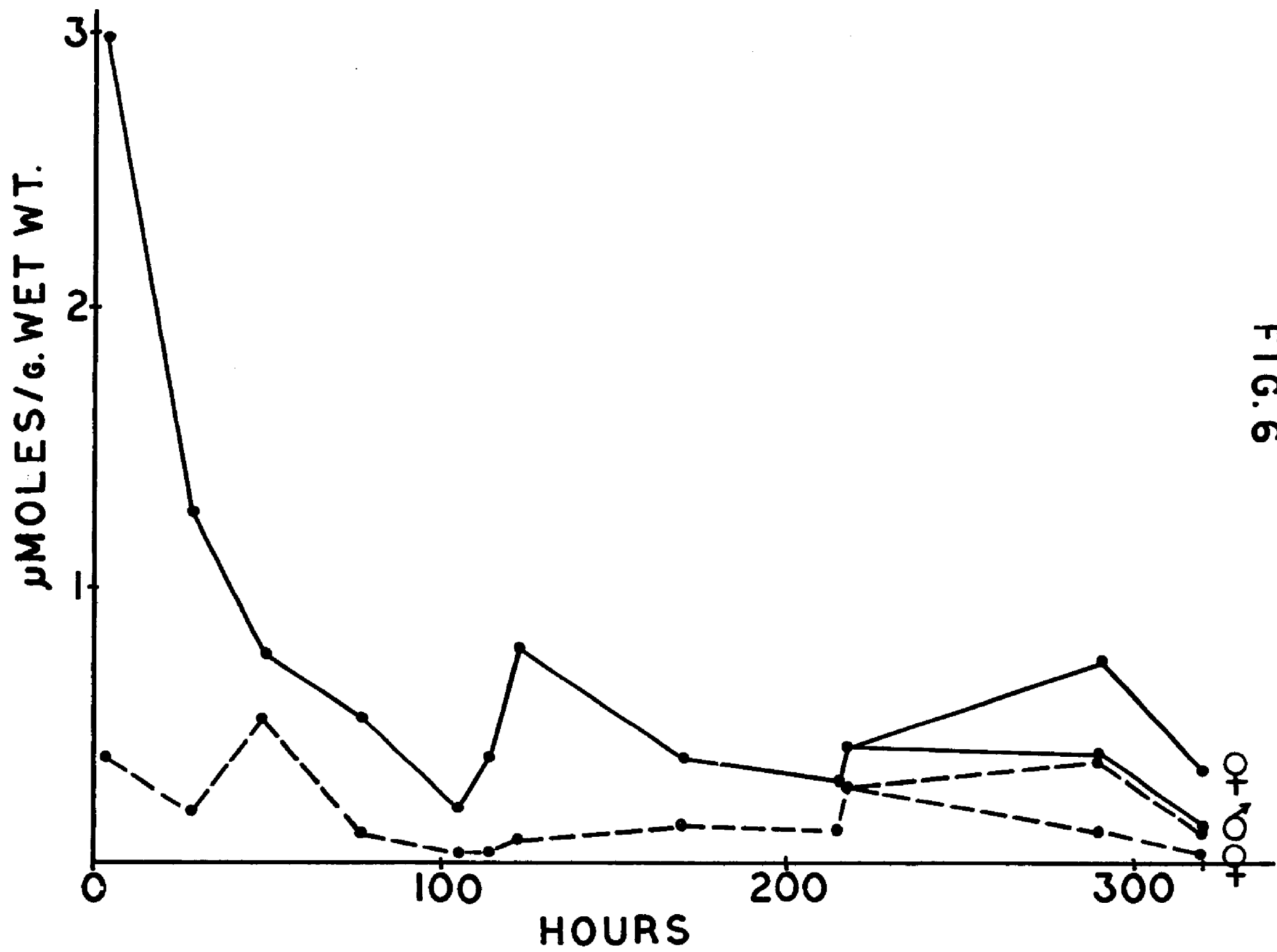


FIG. 6

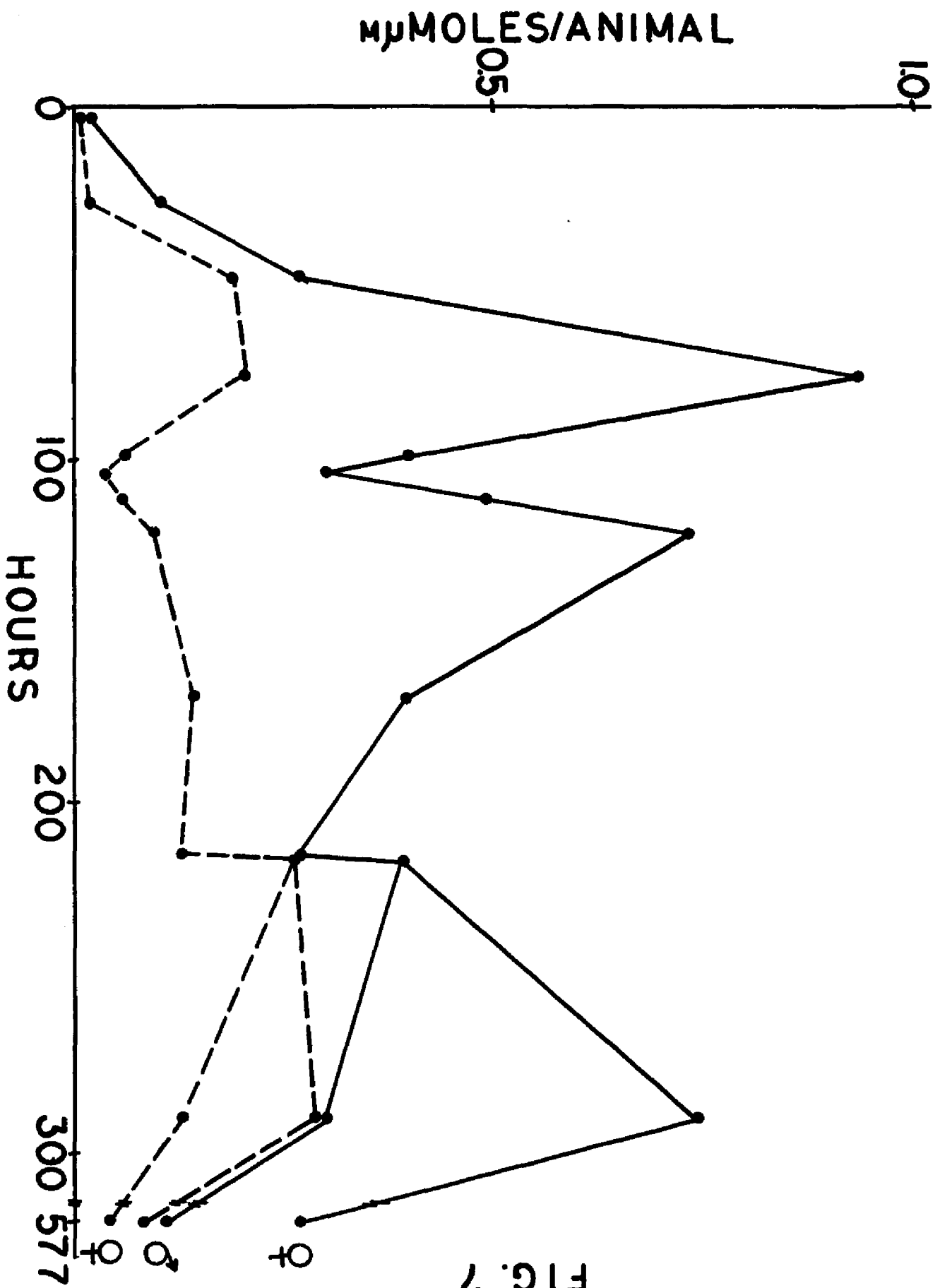
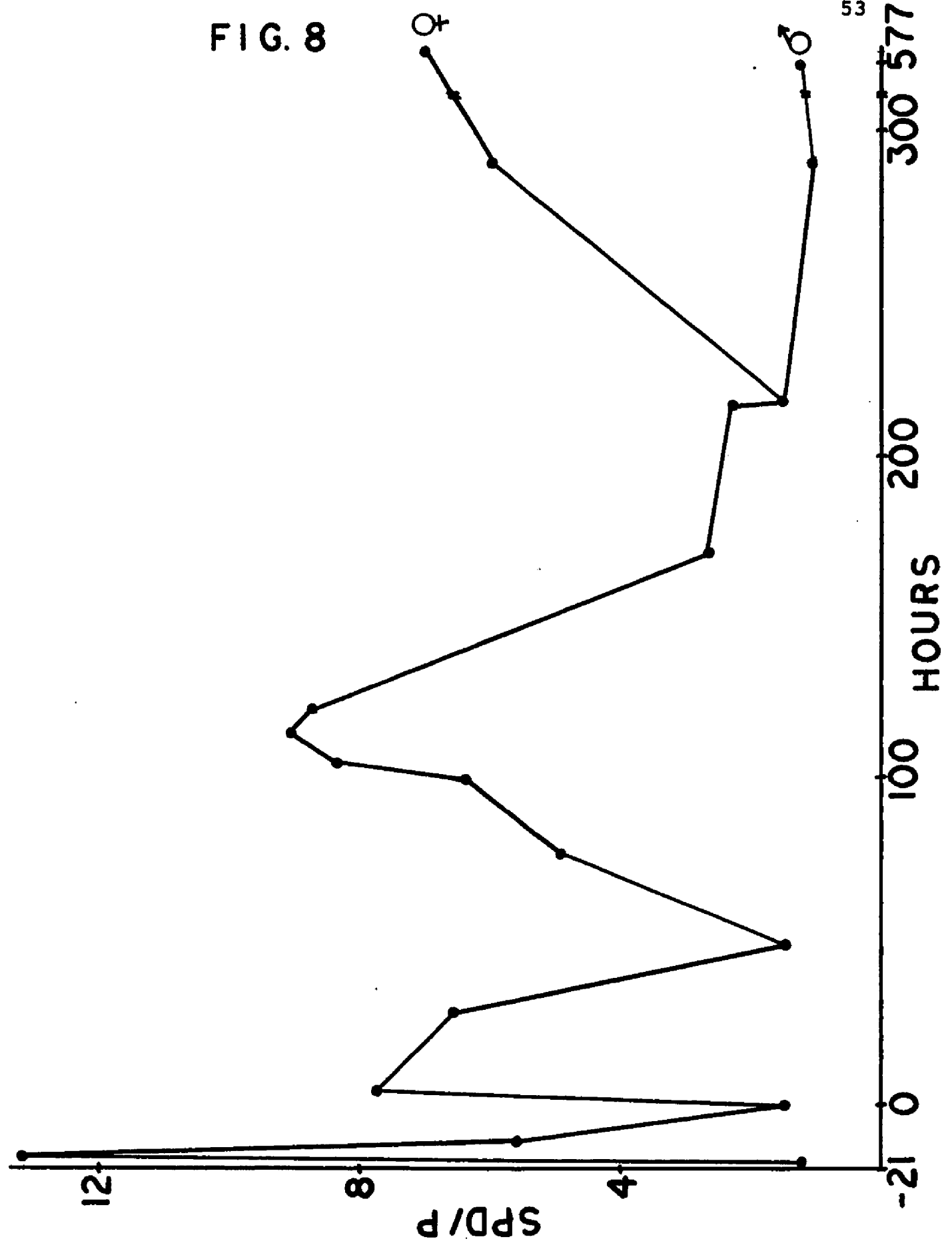


FIG. 7

Figure 8. Spermidine/putrescine ratios as a function of development at 25° C in D. melanogaster.

FIG. 8



3. PUTRESCINE AND POLYAMINE LEVELS: RELATION TO VARIOUS BIOCHEMICAL GROWTH PARAMETERS

Although not precise, the growth of an organism has long been expressed in terms of wet weight. Figure 9 demonstrates the individual wet weight of the stages of the present stock of D. melanogaster. Fresh egg weight remains quite constant at 0.0071 mg./egg during embryonic development. Beginning with hatching large increases in wet weight are observed, reaching a maximum at 77 hours in third instar larvae. Similarly, Church and Robertson (38) and Nigon and Daillie (115) have observed maximum wet weights at 72 hours and 90 hours, respectively. The decrease in wet weight beginning in the latter part of the third instar presumably coincides with the withdrawal from food in search of a pupation site. Little change in wet weight is observed during the pupal period, and, as indicated, males invariably attained a lower wet weight than females of the same age.

The individual protein contents of the stages of D. melanogaster are shown in Figure 10. As noted by Church and Robertson (38) protein content is closely correlated with wet weight up to the time that maximum wet weight is achieved. However, it should be noted that higher protein contents per individual were obtained in the present investigation. This discrepancy may be explained by differences in the methods

employed, but it is probably the result of medium differences, since these authors employed an axenic medium. The latter alternative would also explain the incongruous leveling off of protein content at a time when imaginal tissues are rapidly proliferating.

Figures 11 and 12 illustrate the RNA and DNA content, respectively, on an individual basis in the present stock of D. melanogaster. These data are comparable to those obtained by Church and Robertson (38) for a Pacific Stock of D. melanogaster, i.e. a rapid increase in RNA and DNA content during larval development, leveling off during the pupal stage.

The possible antagonism of spermidine by putrescine with regard to RNA synthesis was first suggested by Cohen et al. (41); therefore it was thought of value to attempt to correlate spermidine/putrescine ratios and nucleic acid biosynthesis. As is obvious from Table 2 there is excellent agreement between high rates of nucleic acid synthesis and high spermidine/putrescine ratios (SD/P); however, these relationships should be regarded with caution, since the level of putrescine could simply reflect the level of biosynthesis and degradation of spermidine. That is, one could have correlated high spermidine content and high nucleic biosynthesis (40,56,130) without invoking putrescine antagonism.

Church and Robertson (38) observed a threefold increase

Table 2. Correlation of spermidine/putrescine ratios and rate of nucleic acid biosynthesis.

<u>TIME (HRS):</u>	<u>STAGE:</u>	<u>SD/P*:</u>	<u>RNA SYNTHESIS:</u>	<u>DNA SYNTHESIS:</u>
4	U. Egg	low	Assumed negligible	
3-8	Egg	high	high	high
20	"	low	low	low
3-28	Larval	high	high	high
49	"	low	low	low
77	"	high	high	high
105	"	high	Rapid proliferation of imaginal tissue.	
Early	Pupal	high	high	high
Mid-& Late	"	low	low	low
Female	Adult	high	high	high
Male	"	low	low	low

* SD: spermidine

P: putrescine

' Unfertilized egg

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Figure 9. Individual wet weight (mg) as a function of time (hours) and development at 25° C in D. melanogaster.

Figure 10. Individual protein content (mg) as a function of time (hours) and development at 25° C in D. melanogaster.

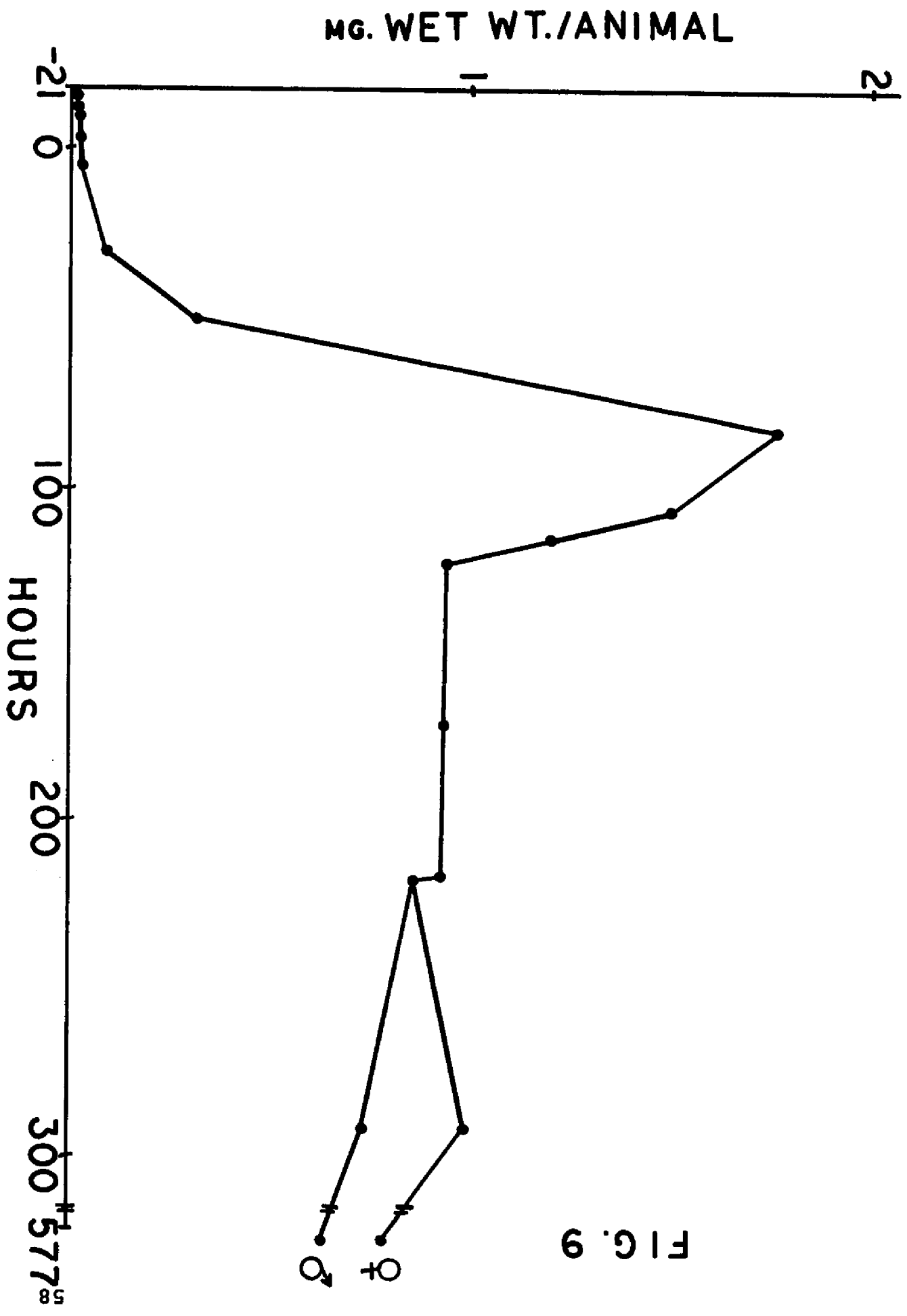


FIG. 9

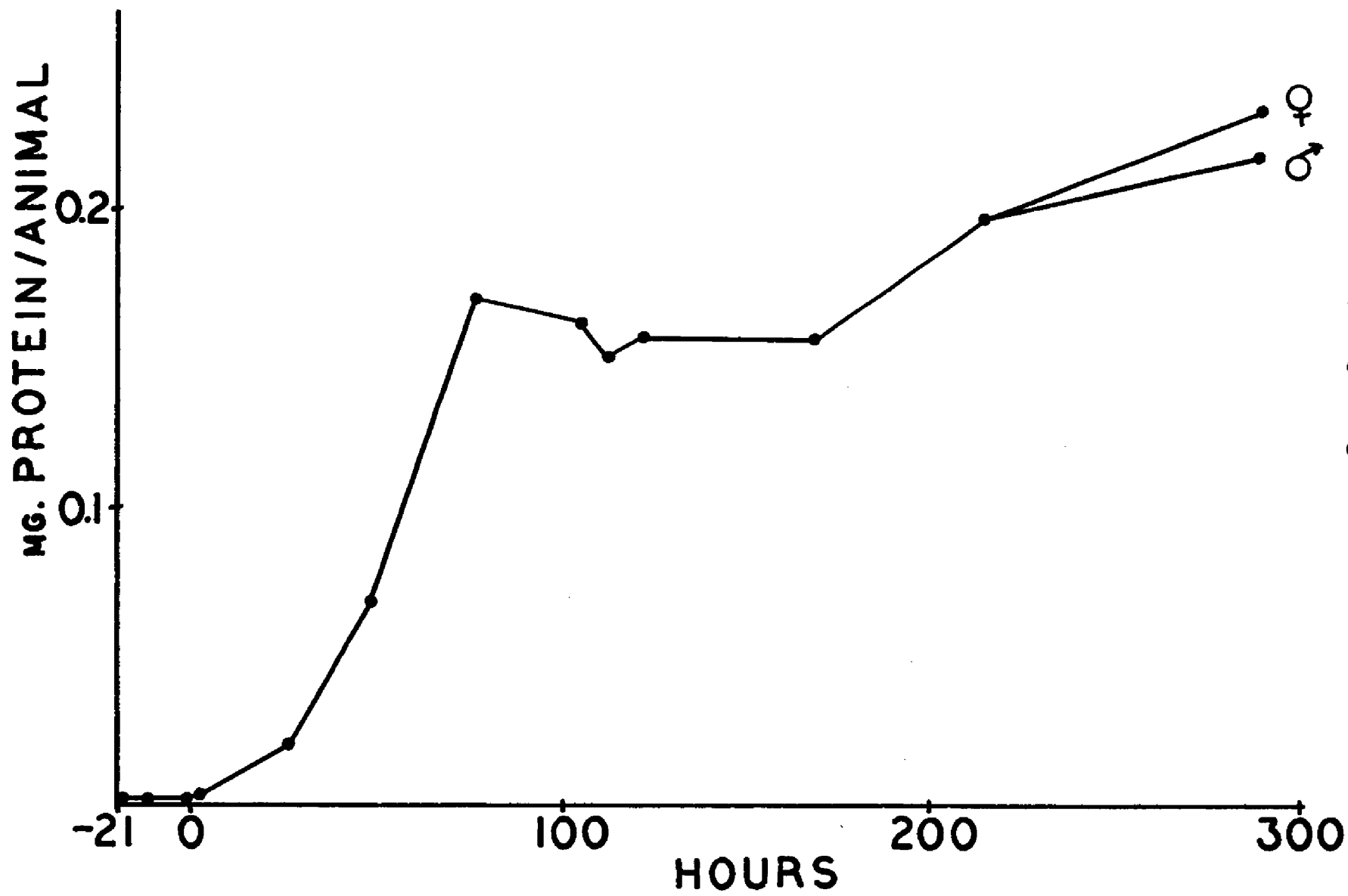


FIG. 10

in total RNA/egg within the first 9 hours of incubation. In contrast Travaglini and Schultz (164) reported a fairly constant content (0.078 $\mu\text{g}/\text{egg}$) of total RNA in embryonic development; however, 4S RNA was observed to increase from 5% to 10% of the total RNA. DNA content /egg has been shown by Nigon and Daillie (115) to increase substantially only during the first half of embryonic development; similar results were obtained by Church and Robertson (38).

High rates of RNA and DNA synthesis have been reported during early larval development, reaching a maximum at 24 hours (38). These same authors reported that nucleic acid biosynthesis was minimal at 48 hours, and again rapidly increased at 72 hours. The late larval period is characterized by the rapid proliferation of imaginal tissues (68).

Although investigations concerning RNA and DNA synthesis in the pupal stage are scanty, it is probably safe to assume high rates of RNA and DNA synthesis in the prepupal and early pupal stages under the influence of the molting hormone, ecdysone (39). That no substantial net gains in RNA and DNA content are observed, is probably the result of extensive larval tissue histolysis. The investigation of RNA and DNA levels in adult Drosophila has demonstrated that only the female undergoes an extensive maturation process involving RNA and DNA synthesis, which lasts for weeks (25). No such phenomenon was observed in males.

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Figure 11. Individual RNA content (μg) as a function of time (hours) and development at 25° C in D. melanogaster.

Figure 12. Individual DNA content (μg) as a function of time (hours) and development at 25° C in D. melanogaster.

Composite data obtained by Church and Robertson (38), Nigon and Daillie (115), and by the present investigation.

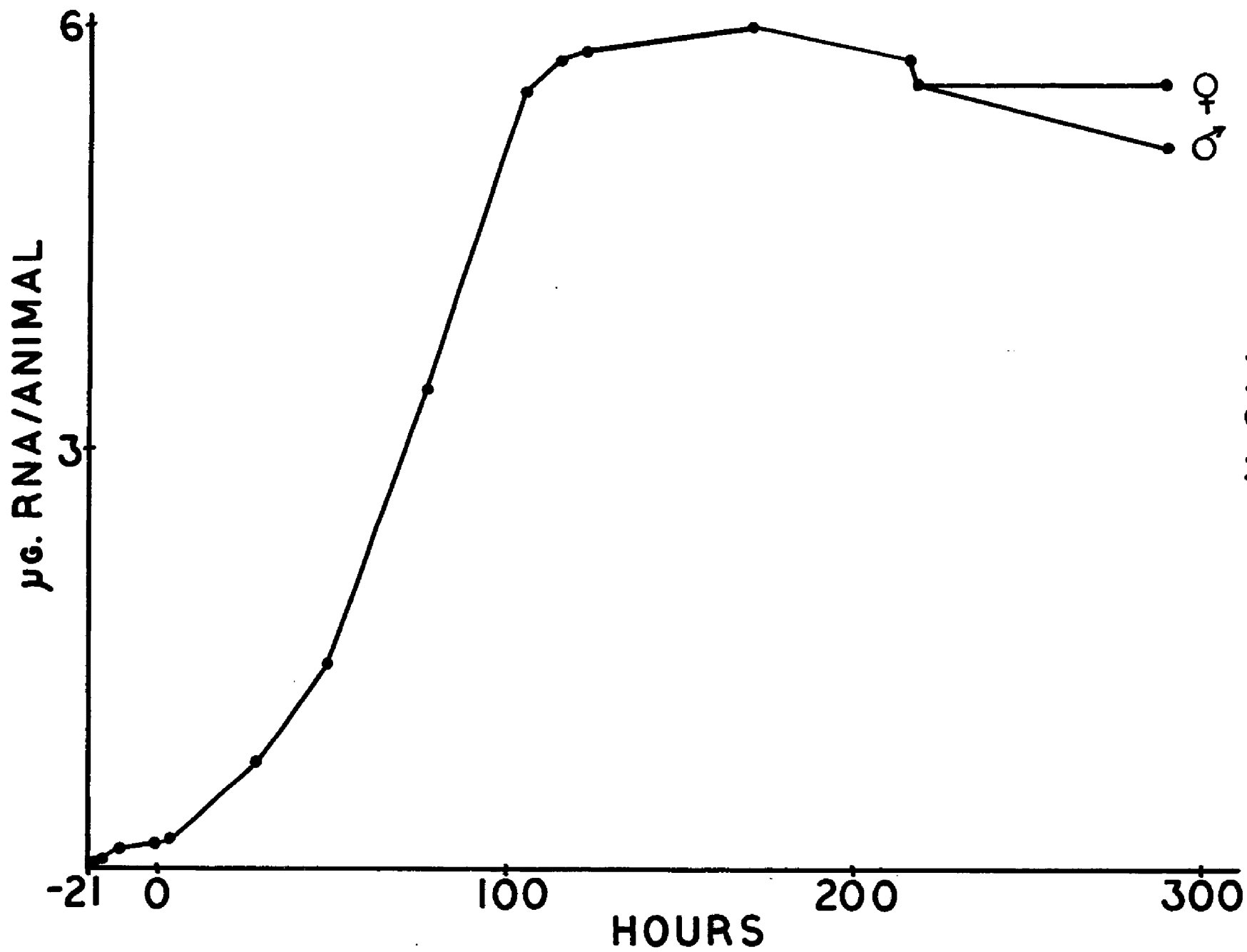


FIG. 11

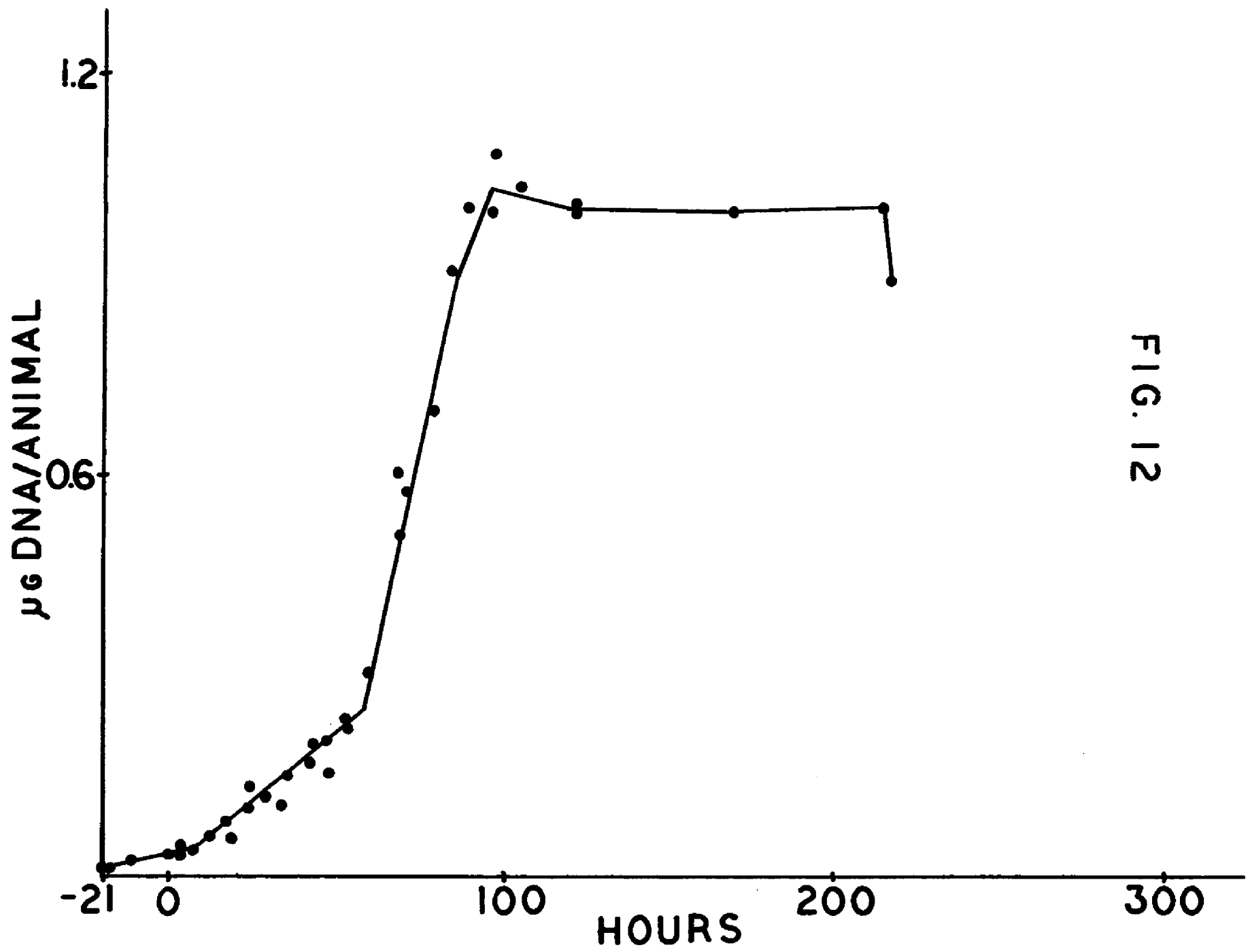


FIG. 12

The stabilization of nucleic acids by polyamines has often been suggested as the mechanism by which these compounds enhance nucleic acid synthesis in vitro. Other investigations, previously cited, suggest polyamine involvement in the release of the RNA product. Recently, Petersen et al. (127), employing doubly labeled ATP (δ - ^{14}C - and γ - ^{32}P -ATP), have reported that spermidine enhanced the incorporation of both ATP's in a purified in vitro system, suggesting not only an increase in RNA synthesis, but also an augmentation in the number of RNA chains synthesized.

The use of purified DNA templates in in vitro studies of polyamine function may be misleading, since DNA is naturally complexed, at least in eukaryotes, with a "polyamine", viz. histone. It is therefore suggested that the mechanism of polyamine action is mediated through "controlled destabilization" under physiological conditions. That is, the polyamines are capable of effectively competing with histone for binding sites on the DNA template, leading to destabilization of the nucleohistone complex. The unique feature of polyamine-nucleic acid interactions, resulting in stabilization, then preserves template integrity leading to enhanced RNA synthesis. It is further suggested that physiological concentrations of the polyamines do not indiscriminately destabilize nucleohistone, but attack "weak spots" in the nucleohistone complex, which could result from acetylation and/or phosphorylation of histone (6,96).

Evidence for the above mechanism has been presented by Agrell and Heby (5), employing double diffusion in gels. Spermidine and spermine were found to precipitate DNA; however, putrescine and cadaverine never produced precipitation lines, and cadaverine prevented DNA precipitation with low concentrations of spermidine and spermine. Similar competition between polyamines and histone was postulated. Schwimmer (139) has shown that putrescine and cadaverine enhanced DNA synthesis primed by nucleohistone; inhibition was observed when native DNA was used as primer. It was argued that these compounds compete with histone leading to a weakened complex which enhanced priming activity.

4. METABOLISM OF PUTRESCINE AND SPERMIDINE IN
DROSOPHILA MELANOGASTER

In Table 3 are given specific activities for putrescine and the polyamines in 2 hour old larvae cultured on sterile medium as a function of time after the oral administration of 1,4-C¹⁴-putrescine. As can be seen only a small conversion of putrescine to spermidine was observed; no label was found in spermine. Similar results were recorded when 2-C¹⁴-methionine was orally administered. The latter result could be explained on the basis of recent data from a number of laboratories which report that the contribution of label from methionine to spermidine is highly dependent on the concentration of putrescine (88,89,122). Inspection of the putrescine level indicates that axenically reared larvae aged approximately 3 hours contain 0.0021 μ moles/individual; non-axenically reared larvae of the same age contained 0.0031 μ moles/individual. In view of the low incorporation of 1,4-C¹⁴-putrescine into spermidine, which does not appear to be methionine concentration dependent (87), it is proposed that the early larval stage requires preformed spermidine in the diet. Another possibility is that the conversion of putrescine to spermidine is dependent upon a nutritionally complete diet, which was not provided in the present experiment. It should also be noted that a small amount of label

was observed in the origin, which probably represents acetylation or a metabolic product(s).

The fate of C¹⁴-putrescine when orally administered to larvae aged 104 hours is presented in Table 4. Again the level of conversion to spermidine appears to be low. In addition the specific activity of putrescine decreased rapidly. Since the pupal stage is a non-feeding stage, the de novo synthesis of spermidine is required to account for the increase in the level of spermidine found in pre- and early pupae. At least two alternatives are plausible: (1) the synthesis of spermidine in D. melanogaster does not utilize putrescine as a precursor, or (2) spermidine has a high turnover rate. The administration of 1,4-C¹⁴-spermidine to 104 hour old larvae appears to support the latter alternative as illustrated in Table 5. Interestingly, these data indicate the rapid conversion of spermidine to putrescine, and the conversion of putrescine to some unknown metabolite(s). Low levels of spermidine conversion to putrescine have been observed in rat liver (144) and in a Pseudomonas sp. (119). In the latter system spermidine is degraded to putrescine and 3-aminopropionaldehyde.

The oxidation of putrescine and various diamines has been reported by Boadle and Blaschko (29) for the cockroaches, Periplaneta americana and Blaberus discoidalis. The nature of the products was not discussed. Certain predictions can be proposed concerning the nature of the breakdown product of

putrescine in the present system, since little label was found in the origin. The latter observation eliminates acetylated putrescine and the following possible conversion of putrescine to amino acids (58): putrescine \rightarrow Δ' -pyrroline \rightarrow γ -aminobutyric acid \rightarrow succinic semialdehyde \rightarrow succinic acid \rightarrow amino acids. The remaining alternatives indicate a dansyl-reactive compound(s) having no carbonyl group, or a non-dansyl-reacting compound.

Table 3. The fate of 1,4-C¹⁴-putrescine in 2 hour old larvae of Drosophila melanogaster.

Time ¹ (hours):	Specific Activity*		
	Putrescine:	Spermidine:	Origin ² :
1	4534	0	19
2	4598	251	120
3	4159	501	160

* Expressed as dpm/0.01 μ moles/individual

¹ Hours after removal from labeled medium.

² Disintegrations/minute (dpm).

Procedure: Synchronized eggs (19 hours) were dechorionated and sterilized as in Methods and Materials. The eggs were resuspended in sterile Ephrussi-Beadle medium containing 0.5% sucrose to hatching, and allowed to mature for 2 hours. The larvae were then washed and resuspended in the above medium containing 1,4-C¹⁴-putrescine (5 μ c/ml.; 9mc/mmmole; New England Nuclear Corp., Boston, Mass.). The larvae were allowed to ingest the label for 45 minutes, washed in Ephrussi-Beadle medium, and placed on sterile sucrose-salts medium for the indicated times. Putrescine and the polyamines were extracted, dansylated, and quantitated as previously indicated. For radioactivity measurements the fluorescence spots were scraped from the plate, transferred to a small test tube, and the dansylated compounds were eluted with 0.5 ml. ethyl acetate. An aliquot (0.3-0.4 ml.) of the supernatant was

then transferred to a counting vial and counted after the addition of 3 ml. cellosolve and 12 ml. toluene containing POPOP and PPO.

Table 4. 1,4-C¹⁴-putrescine metabolism in late larval, pupal, and adult stages of Drosophila melanogaster.

Time (hours):	Stage:	Specific Activity*		Origin ¹ :
		Putrescine:	Spermidine:	
121.5	pupal	12,384	349	100
193.5	pupal	1,323	254	0
294.5	adult males	80	190	34
294.5	adult females	49	75	62

* Expressed as dpm/mumole/individual

¹ Disintegrations/minute (dpm)

Procedure: Larvae aged 104 hours, cultured on non-axenic medium, were washed in Ephrussi-Beadle medium, and allowed to feed for 1 hour on sterile sucrose-salts medium. The larvae were again washed, resuspended in Ephrussi-Beadle medium containing 0.5% sucrose and 5 µc/ml. 1,4-C¹⁴-putrescine (9mc/mmoles), and allowed to feed for 1 hour. After washing, the larvae were incubated on sterile sucrose-salts medium at 25° C, and the indicated stages were harvested. Quantitation and radioactivity measurements were performed as indicated under Table 3.

Table 5. 1,4-C¹⁴-spermidine metabolism in the late larval, pupal, and adult stages of D. melanogaster.

Time (hours):	Stage:	Specific Activity*		Origin ¹ :
		Putrescine:	Spermidine:	
121.5	pupal	6,669	2,267	727
193.5	pupal	935	664	67
294.5	adult males	83	0	45
294.5	adult females	0	233	309

Procedure: Same as indicated under Table 4, except larvae were incubated in the presence of 1,4-C¹⁴-spermidine (5 μ c/ml.; 2.69 mc/mole; New England Nuclear Corp., Boston, Mass.).

* Expressed as dpm/ μ mole/individual

¹ Disintegrations/minute (dpm)

5. THE EFFECT OF PHYSIOLOGICAL CONDITIONS ON PUTRESCINE
AND POLYAMINE LEVELS IN DROSOPHILA MELANOGASTER

These investigations were begun in an effort to explain the discrepancies which existed between the present data concerning putrescine and polyamine levels in D. melanogaster and those previously published by Dion and Herbst (49). The latter investigation had revealed high putrescine and lower spermidine levels; however, the present investigation indicated a spermidine/putrescine ratio greater than unity for all stages, except for the unfertilized egg. That temperature was responsible for these differences was plausible, since the previous results had been conducted on stages held at ice temperature during collection, and stored at -20° C until assayed. In contrast the present investigation was conducted on stages rapidly collected at ambient temperature, and assayed immediately.

As indicated in Table 6 there is a rapid decline in spermidine and putrescine content in early pupae when held at ice temperature for the indicated times, which cannot be accounted for by the increase in spermine observed at 1 hour. Although not assayed experimentally in this system, these results probably can be explained by the observation of Tabor (153), viz. that low temperatures effect a rapid acetylation of spermidine in E. coli. However, these observations still do not explain a spermidine/putrescine ratio

less than 1 in stages stored at -20° C. It is therefore suggested that short term exposure to low temperatures results in the acetylation of putrescine and spermidine, but that long term exposure probably results in the conversion of spermidine to putrescine. In relation to the homeostatic control over putrescine and spermidine levels and their effect on RNA synthesis, it is envisioned that deacetylation of putrescine and spermidine would be less energy requiring than the conversion of putrescine to spermidine. Also it is proposed that a spermidine/putrescine ratio less than 1 would be more effective in decreasing RNA synthesis than would be a decrease in both of these compounds while maintaining a similar ratio.

Table 7 illustrates the effect of heat shock, i.e. increasing temperature 10° C, on putrescine and polyamine levels in synchronized larvae aged 100 hours. This procedure has been utilized for some time to induce puffing, i.e. the activation of certain gene sites in polytene chromosomes. (See Dion, 47, for review of literature dealing with gene activation in polytene chromosomes.) Reported data (26, 27, 168) indicate that heat shock activates a specific set of genes within a short period of time, which are usually present in development only just prior to puparium formation in Drosophila. It is important to note that the level of spermidine rises (33%) within 20 minutes, and that this

15

level is not normally attained until some time after white puparium formation and before pupation. Thus it is suggested that spermidine is capable of controlling RNA synthesis by effectively competing with specific histones for specific gene sites, resulting in puffing. That the level of spermidine is capable of controlling puff size and therefore RNA synthesis, is indicated by data (26) which demonstrate a regression of temperature induced puffs after 20 minutes, e.g. in stomach cells of D. hydei. A similar pattern is observed in the level of spermidine in Table 7.

It has been proposed by Dion and Herbst (49) that the insect molting hormone, ecdysone, influences the concentration of spermidine in the prepupal stage, which is characterized by a high ecdysone titer. That ecdysone influences putrescine and spermidine levels is illustrated in Table 8 for larvae aged 75 hours. The concentration of putrescine rose within the first hour and decreased thereafter; spermidine displayed a more complex pattern, decreasing at 1 hour, rising at 2.5 hours, and again decreasing at 5.0 hours. However, these data are complicated by a number of factors. Although ecdysone effects on development have been observed by oral administration (61,62), higher concentrations are necessary when compared to ecdysone applied by injection. In addition the concentration of ecdysone is critical in obtaining maximal puffing, and, therefore, optimal RNA synthesis. Clearly, additional experimental data employing

various ecdysone concentrations and modes of application are required. Finally, it should be noted that the oral administration of ecdysone to late larvae (approximately 105 hours old) was without effect on putrescine and polyamine levels.

Table 6. The effect of low temperature on putrescine and polyamine levels in early pupae of Drosophila melanogaster.

Time (hours):	Putrescine*:	Spermidine:	Spermine:
0	0.1288	0.8067	0.0611
1	0.0988	0.6700	0.1245
2	0.0560	0.5967	0.0789
3	0.0933	0.6021	0.0945

* μ moles/individual

Procedure: Early pupae were washed in Ephrussi-Beadle medium and placed in test tubes contained in an ice bath for the indicated times. Putrescine and polyamines were assayed as previously described in Methods and Materials.

Table 7. The effect of heat shock on putrescine and polyamine levels in the late larval stage of Drosophila melanogaster.

Time (min.):	Putrescine*:	Spermidine:	Spermine:
0	0.0634	0.4030	0.0194
20	0.0466	0.5370	0.0272
40	0.0272	0.4530	0.0155
60	0.0233	0.4420	0.0155

* mpmoles/individual

Procedure: Synchronized 100 hour old larvae, cultured at 25° C, were washed and placed on sterile sucrose-salts medium. The larvae were then placed in a 35° C incubator for the indicated times. Putrescine and polyamine levels were assayed as previously described in Methods and Materials.

Table 8. The effect of ecdysone on putrescine and spermidine levels in larvae of Drosophila melanogaster.

Time (hours):	Putrescine*:	Spermidine:
0	0.0933	0.8006
1.0	0.1632	0.2330
2.5	0.1321	0.6804
5.0	0.0778	0.4042

* Expressed as μ moles/individual

Procedure: Larvae aged 75 hours were washed in Ephrussi-Beadle medium, and transferred to sterile sucrose-salts medium for 1 hour. The larvae were again washed and incubated at 25° C in Ephrussi-Beadle medium containing synthetic ecdysone (generous gift of Syntex Corp., Palo Alto, Calif.) at a concentration of 5 μ g/ml. for the indicated times. Putrescine and spermidine were assayed as previously described.

6. ISOLATION AND PARTIAL CHARACTERIZATION OF THE SEX PEPTIDE OF DROSOPHILA MELANOGASTER

The primary purpose of this investigation was to determine whether the unknown component in the sex peptide as reported by Fox (65) was a diamine or polyamine or a derivative of such compounds. The occurrence of large quantities of polyamines in some prostatic secretions, and the possible role of these compounds in fertilization made these compounds likely possibilities. Although these compounds were not found to occur in the sex peptide, this peptide was found to be interesting and deserving of further experimental analysis.

Two dimensional TLC analyses of 80% methanol extracts of male and female Drosophila demonstrated a number of sex differences as illustrated in Figure 13. The chromatogram obtained for females was identical to Figure 13 except for the absence of spots #10 and #15. An additional difference, previously noted by Kaplan et al. (91), was the observation of higher quantities of methionine in females as compared to males. Since spot #10 occurred only in males, and had R_f values closely approximating those reported (63), it was tentatively identified as the sex peptide.

Molecular sieving was attempted to remove the bulk of the amino acids from the sex peptide. Figure 14 demonstrates the elution pattern obtained from an 80% methanol extract of

2.0 g. of frozen males. TLC analyses, as in Figure 13, of the pooled and lyophilized peak samples disclosed that tubes 76-81 contained spots #10 and a portion of spot #11. Preparative cellulose TLC in n-butanol/acetic acid/H₂O (4:1:1, v/v) and elution were then employed to purify the sex peptide.

An ultraviolet scan of the purified material did not suggest the occurrence of an aromatic residue within the peptide. Also amino-terminal analysis by dansylation, followed by acid hydrolysis revealed only the presence of dansyl ethanolamine. The latter observation indicates that some of the ethanolamine residues are covalently bonded to the peptide probably via ester linkage through the alcohol group, and that the peptide does not have a free N-terminus. That diamines or polyamines were not present in the peptide, was evident from a TLC analysis of the products obtained after acid hydrolysis and dansylation, in that order.

Acid hydrolysis of the sex peptide, when analysed by thin layer electrophoresis and chromatography, gave a separation pattern as in Figure 15. As can be seen these data are consonant qualitatively with those published by Fox and Sweeney (65). The presence of methionine sulfoxide could represent the oxidation of methionine during acid hydrolysis. Another possibility is that the former is actually a constituent of this peptide, since no free methionine was detected. From TLC and a preliminary amino acid analysis large amounts of ethanolamine, as compared to the

FIG. 13

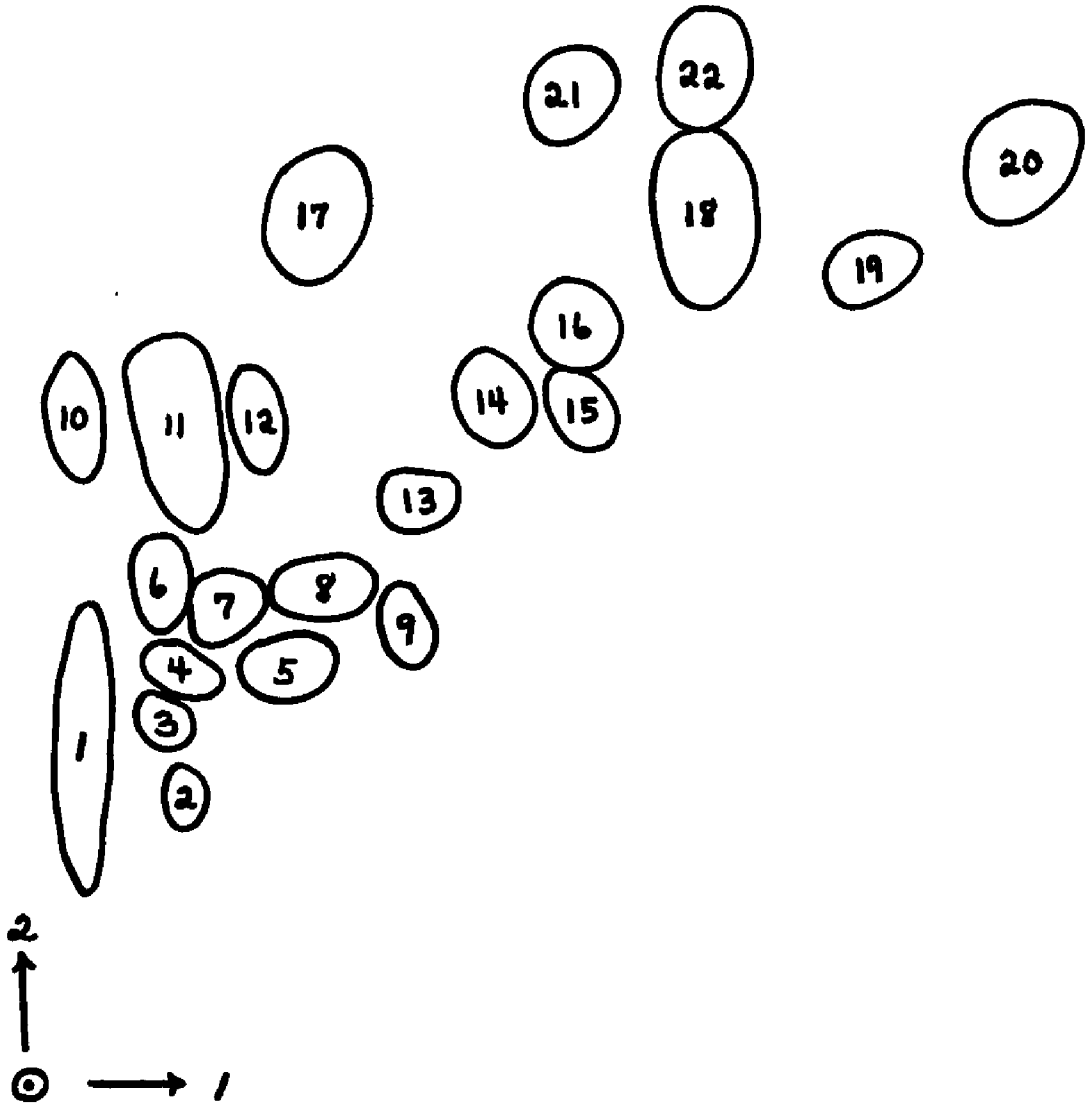


Figure 14. Elution pattern of an 80% methanol extract of Drosophila males applied to a Sephadex column as described in Methods and Materials. 2.15 ml. fractions were collected at a flow rate of 12 ml./hour. Approximately 78 ml. were allowed to pass through the column prior to collection.

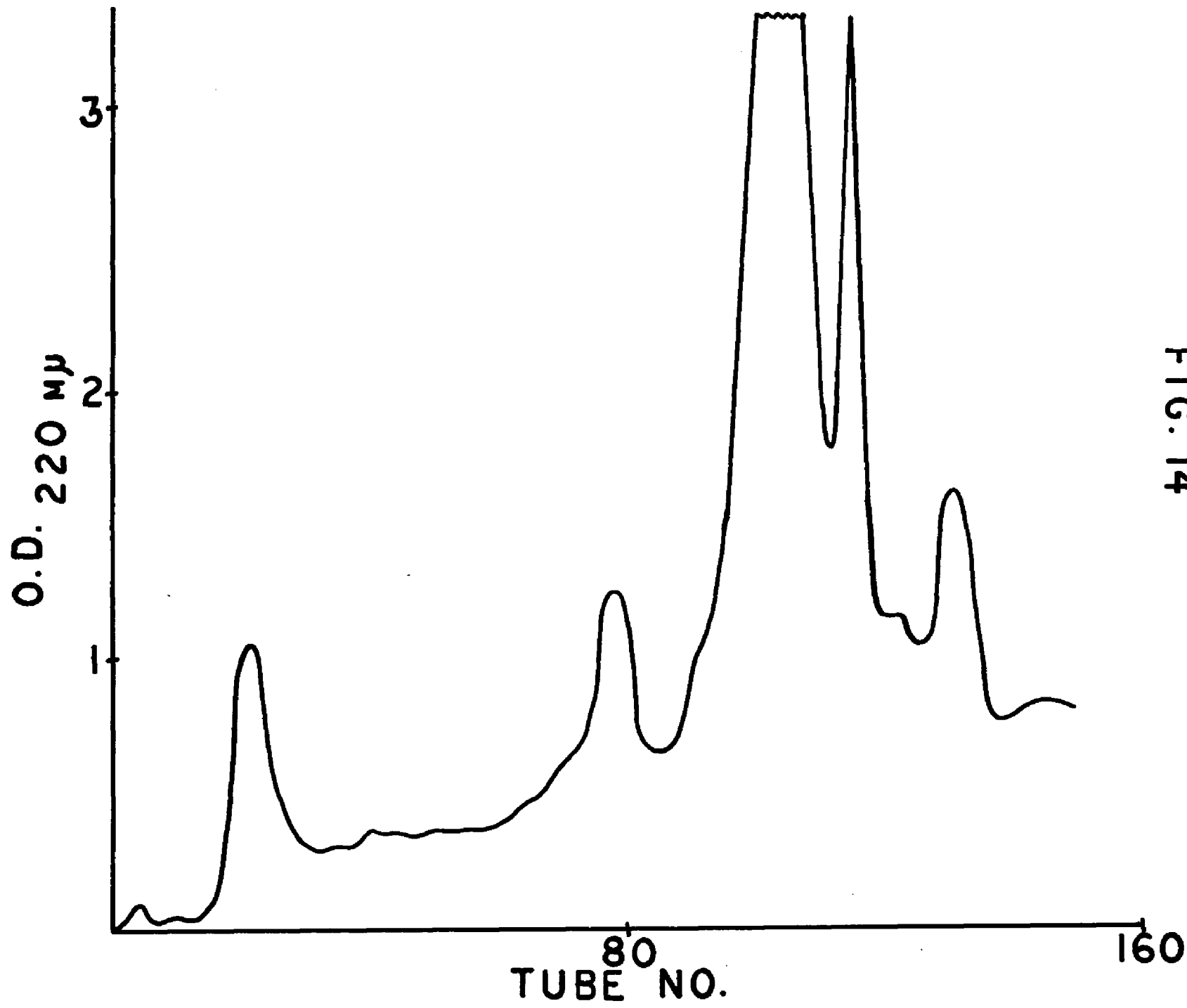


FIG. 14

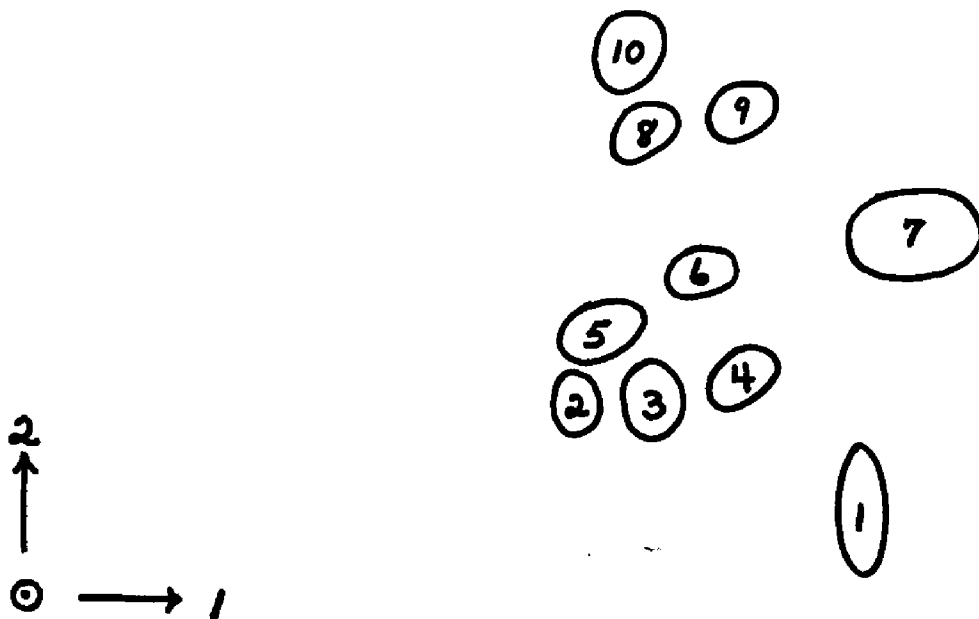
other residues, were found. That this large amount of ethanolamine is not due to impurities, is based on the following. The sex peptide gave a single ninhydrin-positive spot when chromatographed by cellulose TLC in 80% phenol and in n-butanol/acetic acid/H₂O (4:1:1, v/v). A single spot was also obtained when this peptide was electrophoresed in 1.64 M formic acid (pH 1.72) and in pyridine-acetate buffer (133 ml. pyridine and 4.6 ml. glacial acetic acid diluted to 2 l., pH 6.5). It should be noted that the sex peptide was neutral in the latter buffer. One ninhydrin-positive spot was also obtained on chromatography on silica gel G layers in 90% acetic acid; however, charring in 50% H₂SO₄ for 30-60 minutes at 100° C revealed two additional components. Since the latter were ninhydrin-negative, they probably do not represent phosphatidyl ethanolamines. Purification of the sex peptide by preparative TLC on silica gel H layers followed by acid hydrolysis, still revealed high concentrations of ethanolamine.

Analysis of the trace amount of the unknown component indicated that it was basic, i.e. migrated toward the cathode in pyridine-acetate buffer (pH 6.5) with approximately the same mobility as lysine. This component was also found to be Pauly reagent negative, eliminating histidine and some of its derivatives. Since an amino acid analysis of the sex peptide hydrolysate revealed the presence of small amounts of ornithine and lysine, the unknown is tentatively identified as a mixture

Figure 15. Separation pattern of acid hydrolysate of the sex peptide of Drosophila males. First dimension: TLE (300 volts, 7.0 mA for 45 min.) in 1.64 M formic acid, pH 1.72. Second dimension: n-butanol/acetic acid/H₂O (4:1:1, v/v).

Spot #:	Compound:
1	unknown
2	aspartic acid
3	serine
4	glycine
5	glutamic acid
6	α -alanine
7	ethanolamine
8	methionine sulfoxide
9	valine
10	leucine

FIG. 15



of these compounds.

Enzyme digestion studies employing carboxypeptidase A, trypsin, and chymotrypsin according to the methods of Margoliash (106) revealed that the sex peptide is resistant to all of these treatments. Resistance to carboxypeptidase A and the apparent lack of an N-terminus strongly favors the conclusion that the sex peptide is cyclic or that both the C- and N-termini are substituted. Lack of digestion by trypsin supports the argument that ornithine and lysine are trace components. That no digestion with chymotrypsin was observed, indicates that the secondary sites of attack, viz. leucine and methionine, are probably "buried", i.e. the peptide assumes a conformation impervious to enzymatic attack.

The above data clearly suggest that the sex peptide is a complex structure. The covalent binding of large amounts of ethanolamine is especially difficult to explain, since amide and phosphate diester linkage of ethanolamine to glutamic and aspartic acids and serine, respectively, requires much larger quantities of these compounds than have been observed. That phosphate diester and/or phosphonamide linkage is involved can be assumed from the observation that Drosophila males fed radiophosphorous contain large quantities of this compound in the paragonia and that females become radioactive upon insemination (95); however, exclusive linkage of this type would probably not result in a neutral peptide.

Preliminary investigations on another peptide which also occurs only in males, spot #15, disclosed that this peptide was qualitatively similar to the sex peptide; however, this peptide contained large quantities of glycine in lieu of ethanolamine. The possible precursor relationship of this peptide to the sex peptide is proposed. The precursor peptide is then envisioned as essentially a glycine polymer, which is reduced to form the sex peptide. Acid hydrolysis of the sex peptide would then yield ethanolamine.

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APPENDIX

Stage:	Putrescine*:	Spermidine:	Spermine:	Spd/P:
Egg				
U.F. ¹	0.0395	0.00385	trace	0.097
1.5 h	0.0171	0.0197	"	1.15
3.0 h.	0.0005	0.0055	"	11.46
5.0 h.	0.0006	0.0079	"	13.17
8.0 h.	0.0013	0.0072	"	5.54
20.0 h.	0.0088	0.0127	"	1.43
Larval				
3.0 h.	0.0031	0.0239	0.0019	7.71
28.0 h.	0.0160	0.1052	0.0119	6.58
49.0 h.	0.1826	0.2660	0.0357	1.46
77.5 h.	0.1920	0.9436	0.2018	4.92
100.0 h.	0.0634	0.4030	0.0194	6.35
105.0 h.	0.0370	0.3100	0.0124	8.37
114.0 h.	0.0551	0.4862	trace	8.83
Pupal				
122.0 h.	0.0843	0.7379	0.1003	8.75
170.0 h.	0.1469	0.3859	0.0136	2.63
215.0 h.	0.1239	0.2765	0.1530	2.23
Adult				
217.0 h.	0.2599	0.3850	0.0308	1.48
291.0 h. F ²	0.1258	0.7408	0.2283	5.89
291.0 h. M ³	0.2858	0.3024	0.3049	1.06
577.0 h. F	0.0386	0.2679	0.0154	6.94
577.0 h. M	0.0770	0.0947	0.0894	1.23

* Expressed as μ moles/organism

² Females

³ Males