Winter 1974

STUDIES WITH DROSOPHILA MELANOGASTER: A THE EFFECT OF POLYAMINES UPON RNA SYNTHESIS B L-ORNITHINE AND S-ADENOSYL-L-METHIONINE DECARBOXYLASE C THERMOLABILITY OF RNA

CRAIG VERNON BYUS

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A. THE EFFECT OF POLYAMINES UPON RNA SYNTHESIS

B. L-ORNITHINE AND S-ADENOSYL-L-METHIONINE DECARBOXYLASE

C. THERMOLABILITY OF RNA

by

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B.A., Johns Hopkins University, 1968

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Biochemistry

February, 1974

This thesis has been examined and approved.

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ACKNOWLEDGEMENTS

I wish to thank Dr. Edward J. Herbst for his guidance during the course of this research. I also wish to express my gratitude to Dr. James Stewart, Dr. Vladimir Glisin, and Dr. Gerald Klippenstein for valuable advice and discussion concerning this work. The excellent technical assistance of Mr. John Wakefield and Mrs. Marilyn Gilbertson is also gratefully acknowledged.

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In order to further elucidate the possible role of polyamines in the synthesis of nucleic acids, a study of the effect of exogenously-administered amines upon the synthesis of RNA in *Drosophila melanogaster* larvae was undertaken. This system was chosen due to the previous investigations relating putrescine and polyamines to growth and development in *Drosophila*.

Larvae cultured on a defined medium containing variable concentrations of spermidine or putrescine accumulated the amines from the media and were enriched with respect to control animals incubated in amine-free cultures. The addition of 1-5 mM spermidine to the liquid culture media resulted in a 30-250% increase in the incorporation of ^{3}H-uridine into 4S, 18S, and 28S RNA from 72-hour *Drosophila* larvae incubated for twenty minutes to twelve hours. 1-100 mM putrescine and 120 mM spermidine inhibited the incorporation of ^{3}H-uridine.

Spermidine was shown to be specific among putrescine, spermine, cadaverine, and ethylene diamine in being able to increase the incorporation of ^{3}H-uridine into RNA. This increase in incorporation was not due to an elevated uptake and conversion of ^{3}H-uridine to ^{3}H-uridine triphosphate or to a more rapid turnover of the RNA.

1-5 mM spermidine had no effect upon the incorporation of ^{3}H-thymidine into DNA of 72-hour Drosophila larvae and slightly increased the incorporation of ^{14}C-L-leucine into total protein. The effect of spermidine on the labeled thymidine triphosphate and leucine pool was also studied.

Assay conditions for L-ornithine decarboxylase (E.C.4.1.1.17) and S-adenosyl-L-methionine decarboxylase in Drosophila larvae were developed. The enzymes were found in high activities and had properties similar to those observed in other tissues. However, in contrast to other tissues, Drosophila ornithine decarboxylase activity was not stimulated by amino acids, and S-adenosyl-L-methionine decarboxylase from 24-hour larvae was only slightly increased by putrescine. The variation in ODC and SAMD activity during larval development is discussed in relation to polyamine accumulations.

The larger species of rRNA from Drosophila larvae was shown to be thermolabile and separated into two "18S" products upon mild heat treatment. The RNA from chick polyribosomes and from Drosophila larvae was co-extracted with phenol, and the chick polyribosomal RNA remained thermostable. Larval 28S rRNA extracted without phenol was also found to be thermostable.

A new procedure for the extraction of RNA was developed. RNA was isolated from several sources free of DNA and protein on a CsCl gradient. RNA extracted in this manner showed a tendency to aggregate yielding an increased proportion, compared to phenol-extracted preparations, of RNA sedimenting as 28S rRNA relative to 18S rRNA. Possible causes for this aggregation were investigated.

Purified 18S rRNA from D. melanogaster and chick polyribosomes aggregated to form new distinct species of RNA following centrifugation through a CsCl gradient. The "18S" products obtained from heated Drosophila 28S rRNA annealed to form a 28S RNA after passage through a CsCl gradient.

LITERATURE REVIEW

A. Polyamines and RNA Synthesis

The polyamines, spermidine ($NH_2(CH_2)_3NH(CH_2)_4NH_2$) and spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$), and the diamine, putrescine ($NH_2(CH_2)_4NH_2$), are nitrogenous bases which have been ubiquitously found throughout the plant and animal world (1). Since the observation of Herbst and Snell (2) that these amines were essential for the growth of certain microorganisms, much has been done to establish their biological roles. At neutral pH, the nitrogen groups possess a unit positive charge and have a strong affinity for intracellular anions such as nucleic acids. It has been the relationship between polyamines and nucleic acids, especially to RNA and RNA synthesis, which has given new impetus to the study of polyamines in the past decade. The metabolism and biological functions of polyamines have been extensively reviewed to date in three symposia (3-5) and one book (6), all published since 1970.

The evidence linking polyamines to RNA synthesis is basically of two types. Indirect evidence involves studies made of the temporal relationship during development between polyamine levels; the activities of their biosynthetic enzymes, S-adenosyl-L-methionine decarboxylase (SAMD) and ornithine decarboxylase (ODC); and the net accumulation and rate of synthesis of RNA. Such observations are most often made during times of rapid growth of an organism, when RNA synthesis

is generally high. This can be at an embryonic or larval stage of development or can involve the cellular response to a specific stimulus; i.e., stimulation of rapid cell proliferation by hormones and drugs or the regenerative response elicited by organ excission. The direct studies are concerned with the effect of exogenously administered polyamines upon the rates of nucleic acid synthesis in a particular organism cell-type.

The relationship of putrescine and polyamine levels to nucleic acid synthesis and accumulation in Drosophila melanogaster was extensively studied by Herbst and Dion (7,8) in this laboratory. They observed that a rise in the concentration of spermidine occurred during periods of embryonic, larval, pupal, and adult development concurrent with increased rates of RNA and DNA synthesis. Putrescine was also present in Drosophila while spermine was barely detectable. A recent study of polyamine variations during the development of the blowfly, Calliphora erthrocephala (9), supports the observations that, in insects, spermidine is the amine most likely to be related to nucleic acid metabolism.

Caldarera and Moruzzi (10) investigated polyamines and nucleic acid metabolism and found the highest concentrations of spermidine, spermine, and putrescine at day four of development, when the RNA concentration also reached a maximum. The level of putrescine fell to a low value by day twenty, and the levels of spermidine and putrescine reached a peak at day twelve--thirteen corresponding to a similar rise in the nuclear and ribosomal RNA concentration.

Other investigators have shown a six-fold increase in ODC (11) and SAMD (12) activity from day two--five in the chick embryo. The enzyme activities then fell off markedly as did the rate of RNA synthesis.

Significant levels of putrescine have also been found in mammalian embryonic tissue. In the fetal rat putrescine and spermidine concentrations reached their highest point at day thirteen, preceding by three days a similar peak in the level of RNA (13). The activities of ODC and SAMD were seen to rise dramatically (100 X) prior to the increase in amine levels and then to decline to a low value between days eleven and fifteen of embryonic development (13). It is interesting to note that elevated histamine synthesis thought to be important in rapid growth did not occur until one day after the maximal RNA level.

In the developing embryo of Xenopus laevis, the African clawed toad, there exists a similar relationship between putrescine and spermidine levels and RNA synthesis. However, this pattern is quite different in an anucleolar mutant which is incapable of synthesizing ribosomal RNA. The putrescine and spermidine concentration in the mutant during the latter stages of development is one-half the levels found in the normal embryo. The ODC activity in the animals lacking nucleoli was reduced 100 times, and the normal SAMD activity was absent. The activity of other enzymes known to increase in the normal embryo during this period of development increase in the mutant also (14).

In developing insects, birds, amphibians, and mammals, there is a definite correlation between the patterns of synthesis and accumulation of polyamines and nucleic acids. Such relationships have also occurred following the application of growth stimuli to non-growing eucaryotic systems.

Following partial hepatectomy of the rat, maximal concentrations of putrescine and spermidine occurred twenty-four hours before the rate of RNA synthesis reached its highest value. The activity of SAMD also increased three times during this period (15). ODC activity rose to ten times the pre-hepatectomy level (16) and was seen to be the earliest consistent indication of the stimulation of cell growth in a variety of tissues (17). Enzymatic decarboxylation of other amino acids existed at the same levels in both sham-operated and hepatectomized animals (17).

ODC activity increased four--five times following kidney hypertrophy in the rat. Major increases in polyamine levels occurred during the first three hours of compensatory growth paralleling increased accumulation of RNA (18).

Jänne et al. have shown that treatment of rats with growth hormone resulted in a stimulation of RNA and protein synthesis. Such treatment also causes an increased level of putrescine and spermidine and a four-fold rise in ODC activity (19). These hormone-induced changes were partially reversed by the injection of either actinomycin D or puromycin. Hepatic ODC activity in adrenalectomized rats was also increased by a variety of other hormones including hydrocortisone, insulin, glucagon, and thyroxin (20).

De novo synthesis of spermidine and nuclear RNA began at the same time in the uterus of the castrated rat following administration of estradiol-17β (21). The concentrations of putrescine and spermidine increased two--three times following greatly elevated levels of ODC and SAMD activity. In the immature rat uterus, ODC activity was stimulated also by a series of non-steroidal estrogens (22).

Seiler and Askar (23) showed that administration of perfluorovaleric acid to mice resulted in a dramatic rise in the level of liver RNA and in the concentration of spermidine.

Wyatt et al. (24) investigated hormonal stimulation of insect metabolism and polyamine accumulation. A large increase in the activity of ODC coincided with a general stimulation of RNA synthesis following injection of β-ecdysone in the diapausing silkmoth. In contrast to what was seen in other tissues, the maximal spermidine accumulation did not occur until after the rate of RNA synthesis had begun to decline.

A definite relationship between increased synthesis of polyamines and RNA exists during the early growth stages of tumors, and elevated polyamine levels were found in the urine of human cancer patients (25).

Goldstein (26) investigated the effect of exogenously applied spermine upon nucleic acid and protein synthesis in cultured Walker 256 carcinosarcoma cells. When spermine was added to the incubation media, there was a 40% and 89% increase above control values in the amount of ^{14}C-uridine

incorporated into total RNA during nine and fifteen-hour labeling times. Protein synthesis was inhibited by 70%, and DNA synthesis remained uneffected.

Sea urchin embryos incubated with ^{3}H-uridine and one mM spermidine showed a 60% increase above control levels in the specific activity of 28S ribosomal RNA (27). Incorporation of ^{32}P-phosphate into rRNA was also increased over a 21-hour period. One mM putrescine did not show any measurable effect upon RNA synthesis.

Caldarera and his co-workers (10,28) observed that the injection of 0.5 μmole of spermine or spermidine into the air space of chick embryos increased the incorporation of ^{3}H-formate by 100-150% into the nuclear RNA and DNA and by 200-250% into the rRNA of the embryonic brain. Spermine caused a 290% rise in the specific activity of total RNA labeled with ^{3}H-uridine. A greater proportion of rapidly-labeled RNA (mRNA) was bound to polyribosomes in the presence of injected polyamines. A general and rapid increase in the specific activity of free nucleotides of chick embryos injected with ^{3}H-formate and ^{14}C-orotate following polyamine treatment was also observed (29).

Spermine (5 mM) and spermidine (10 mM) stimulated the incorporation of labeled orotic acid into total RNA in Ehrlich acites cells (30,31) while putrescine had little or no effect.

Fausto (32) in perfusion studies examined the effects of polyamines upon RNA metabolism in normal and regenerating rat liver. He was able to double the intracellular concentration of spermidine by perfusing the liver for three hours

with one mM spermidine. This increased spermidine level resulted in a 50% increase in the specific activity of nuclear RNA in the normal liver and a 25% increase in the regenerating liver. No change, however, was seen in cytoplasmic RNA. High molecular weight (> 28S) RNA, presumably rRNA precursor, was not detectable unless spermidine was included in the perfusate.

Stimulation of RNA synthesis in mature amphibian oocytes by the microinjection of putrescine has also been shown (33). Synthesis of ribosomal RNA was increased five--twelve times by the injection of 125 nl of 0.8 M and 4.0 M putrescine into each egg. The synthesis of tRNA and rRNA was also increased.

In an autoradiographic study done with Xenopus liver cells, ^{3}H-putrescine initially became associated with the nucleus and, paralleling a similar movement of ^{3}H-uridine, was later found in the nucleolus (34). In a similar autoradiographic study, 0.05-0.1 mM spermidine increased the incorporation of ^{3}H-uridine into isolated salivary glands of D. melanogaster (35). Spermidine was also found to accumulate perferentially in the nucleus.

There are several important possible functions of polyamines which relate directly to any study of RNA synthesis. These will be mentioned briefly.

The association of polyamines and DNA-dependent RNA-polymerase has been studied extensively in in vitro systems using procaryotic enzymes (36,37) and, to a lesser degree, in eucaryotic preparations.

There are two major types of DNA-dependent RNA-polymerase found in the nuclei of the rat (38). Polymerase I is predominately located in the nucleoli and has its maximal activity in the presence of Mg^{++} at low ionic strength. Polymerase II is found in the nucleoplasm and exhibits maximal activity at higher ionic strength with Mn^{++}. Polymerase I is believed to be responsible for ribosomal RNA synthesis while polymerase II has been implicated in DNA-like RNA synthesis.

Barbiroli et al. (39) observed that spermine and spermidine stimulate both the Mg^{++} and Mn^{++}-requiring polymerases in the nuclei of the rat prostate. Stimulation of the Mg^{++} polymerase seems to occur during the early stages of the reaction (1-12 minutes) while the increase in the activity of the Mn^{++} enzyme occurs much later (10-40 minutes).

Raina and Jänne (40) showed that the stimulation of RNA polymerase by polyamines is dependent upon the Mg^{++} concentration in the assay. Spermidine only increased the activity of RNA polymerase in rat nucleoli at sub-optimal Mg^{++} levels. Putrescine, however, enhanced polymerase activity over a wide range of Mg^{++} concentration. RNA polymerase activities were increased in nucleoli isolated in the presence of spermidine due to the elevated concentration of DNA associated with the nucleolus during isolation (41).

Folic acid, thioacetamide, and growth hormone stimulated the activity of RNA polymerase in organs of the intact rat (30,40). Such stimulation was preceded by increased ODC

activity and amine synthesis and followed by an increase in RNA concentration.

Polyamines have been shown by a number of authors to effect the enzymatic degradation of RNA. In broken-cell extracts of E. coli, physiological levels of spermine and spermidine inhibited the autodegradation of ribosomes (42). At higher concentrations, however, this inhibition was reversed. Eight mM spermine and spermidine significantly inhibited the activity of alkaline ribonuclease in cell-free preparations from livers of the normal and hypophysectomized rat (43). Putrescine and cadaverine slightly stimulated ribonuclease activity. Increased binding of rat liver ribosomes to endoplasmic reticulum membranes in the presence of spermine has also been associated with increased ribosomal resistance to attack by ribonuclease (44).

In their studies with cultured Ehrlich ascites cells and isolated rat liver nuclei, Raina and Jänne (30,31) indicated that putrescine, spermine, and spermidine decreased the rate of degradation of pulse-labeled RNA following treatment with actinomycin D. Such an interpretation may not be valid in light of the fact that polyamines might interfere with the ability of the antibiotic to inhibit RNA synthesis (45).

B. Ornithine Decarboxylase and S-Adenosyl-L-Methionine Decarboxylase

Ornithine decarboxylase (E.C.4.1.1.17) catalyzes the decarboxylation of ornithine to yield putrescine. It has been purified about 300 times from rat prostate (46). ODC has a

molecular weight of between 35,000 and 45,000 and a pH optimum of approximately 7.0. The activity of the enzyme is greatly decreased with storage, and for this reason most assays are done on fresh material.

A definite co-factor requirement for ornithine decarboxylase has been established by two methods. Dialyzed tissue extracts only possessed ODC activity after pyridoxal-phosphate was replaced in the assay media (47), and there was a total lack of enzyme activity in the presence of certain inhibitors of pyridoxal-requiring enzymes (48).

Thiols, especially dithiothreitol, stimulate the activity of this enzyme in both crude and purified preparations (46,47). This is due to the fact that in the absence of stabilizing thiols, there is a shift from an active monomeric form of ODC to an inactive dimer (46).

Snyder et al. (11) observed that in the rat liver and chick embryo, approximately 50% of the total cellular ODC activity was isolated with the nucleus. In the normal liver, ODC has a half-life of only eleven minutes--the shortest half-life ever reported for a mammalian enzyme. This half-life can be lengthened by prior treatment with growth hormone (11).

Injection of puromycin increased ODC activity in the rat liver, possibly by causing an increase in amino acid concentration (49). In cultured lymphocytes, increased concentrations of amino acids decreased the rate of degradation of ODC and illustrated a possible post-transcriptional control mechanism for the enzyme (50).

S-adenosyl-L-methionine decarboxylase, when assayed in a crude cellular homogenate, catalyzes the decarboxylation of S-adenosyl-L-methionine and the stoichiometric transfer of the propylamino group to either putrescine yielding spermidine and/or to spermidine to yeild spermine (12). These functions could be accomplished by three separate enzymes--an adenosyl methionine decarboxylase and two propylaminotransferases, one catalyzing the transfer from decarboxylated adenosyl methionine to putrescine (spermidine synthase), and the other, the transfer to spermidine (spermine synthase). It is possible, however, that one enzyme is performing all of these functions.

Jänne was able to dissociate the decarboxylase from the synthases during enzyme purification in the rat prostate (51,52) and the rat liver (53). Russell (54,55), however, was unable to separate the stoichiometric relationship between CO_2 production and spermidine synthesis in a 350-fold purification of SAMD in rat liver.

SAMD was found to have a molecular weight of 50,000 and to have a requirement for pyridoxal-phosphate (55). The decarboxylation reaction has also been shown to be greatly stimulated by putrescine (51-55). No putrescine stimulation, however, was found in Physarum polycephalum (56).

SAMD is confined to the cytoplasm in a number of tissues, including the rat brain (57). The half-life of SAMD is also somewhat longer than that of ODC--60 minutes in the rat liver (14), 120 minutes in the rat kidney (58), and 40 minutes in cultured lymphocytes (59). This half-life was

increased to almost twenty hours by the addition of methylglyoxal Bis (guanylhydrazone), an anti-leukemic agent whose antiproliferative effect can be overcome by the administration of spermidine (58,59).

C. Thermolability of Insect 26-28S rRNA

Applebaum, Ebstein, and Wyatt (60) first noticed in 1965 during the extraction of RNA from Hyalophora cecropia that, if the phenol tissue extract were heated to 50-60° for several minutes, the larger rRNA species (26S) was not visible upon density-gradient centrifugation. On the other hand, the gradient peak of the smaller rRNA species (17S) was greatly increased in size. Subsequent experiments with purified 26S RNA showed that the 26S rRNA was splitting into two pieces whose sedimentation coefficients were identical to the normal 17S rRNA. Since then, this unique thermolability of the insect 26-28S rRNA has been observed in ten different insect orders (see ref. 61 for a complete list) and in the free-living amoeba, Acanthamoeba castellani (62).

The thermal product from the large rRNA of all the species studied has not been separated from the native 16-18S rRNA by sucrose density-gradient centrifugation or by acrylamide gel electrophoresis. Ishikawa and Newbourgh (63), however, reported that 18S "product" in Galleria mellonella does sediment slightly faster than the native 18S rRNA and is found as a single peak following cesium sulfate equilibrium-sedimentation analysis. They also observed that the

nucleotide composition of the thermal product is very similar to 28S rRNA and very different from native 18S rRNA.

There is some confusion in the literature as to whether rRNA precursor is also thermolabile. Petrie et al. (64) found that at least a portion of the 38S and 30S rapidly-labeled precursor in D. melanogaster dissociated into smaller species upon heating. However, the precursor in the silkmoth (60), the waxmoth (63), and in D. virilis (65) all have been reported to be thermo-stable.

The existence of an "extra" 3'-terminus has been proven in 26S rRNA isolated from Aedes aegypti. Using a technique developed to label the free 3'-nucleotide ends of RNA (periodate oxidation followed by incubation with ^{3}H-isonicotinic acid hydrazide) (66), Shine and Dalgarno (61) observed the presence of three nucleotide chains in 26S rRNA, in contrast to the two that are present in most eucaryotic large rRNA species (67,68).

In addition to its thermolability, insect 26-28S rRNA dissociates in the presence of organic solvents known to interfere with hydrogen bonding. Incubation of purified RNA with high concentrations of dimethyl sulfoxide at 0^{o} or room temperature resulted in a similar conversion of 26-28S rRNA to a 17-18S species (60,62,64). Treatment of 28S RNA from the silk gland of the waxmoth with 8 M urea also caused complete conversion to an 18S product (63).

Ionic strength has been found to greatly effect the stability of 26-28S RNA. Petrie et al. (63) stated that an

increased Na^+ concentration in the buffer in which the heating is performed considerably stabilized the rRNA molecules. A ten-fold increase in the salt concentration of the heating buffer caused a 6^o increase in the T_m' (temperature of 50% dissociation) of 26S rRNA isolated from A. aegypti cells (61). A reduction of the ionic strength by lowering the pH or decreasing the buffer salt concentration during either extraction or sedimentation of rRNA from A. castellani resulted in an almost complete disappearance of the 26S rRNA (62).

The effects of heat, DMSO, urea, and ionic strength upon the stability of 26-28S rRNA strongly suggests that hydrogen bonding is playing a major role in preventing the separation of the two major polynucleotide chains. The low temperature and narrow range over which the RNA dissociates (61) indicates that this hydrogen-bonded region is relatively short.

MATERIALS AND METHODS

A. *Drosophila melanogaster* -- Culture Methods

The *Drosophila melanogaster* used for all experiments were originally obtained from Professor Edythe Richardson, Department of Biology, University of New Hampshire. The wild-type flies have been maintained in our laboratory for ten years by repeated sib-matings. The *Drosophila* have been propagated on a synthetic culture media adapted from Pearl *et al.* (69) by the addition of propionic acid. The media was made from two solutions composed of the following ingredients:

Solution A:

sucrose	93.0 g
$NaKC_4H_4O_6\text{-}4H_2O$ (NaK-tartrate)	9.3 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4\text{-}7H_2O$	0.5 g
$CaCl_2$	0.25 g
distilled water to 500 ml	

Solution B:

Bacto-Agar (Difco)	22.4 g
$C_4H_6O_6$ (tartaric acid)	5.0 g
KH_2PO_4	0.66 g
distilled water to 500 ml	

Solution A was stirred until all the solids dissolved, added to Solution B, and the media was slowly heated to 85^o where it turns a clear yellow color. One hundred ml of the hot solution were poured into 500 ml Erlenmeyer flasks

which were closed with clean cotton plugs and allowed to stand overnight at room temperature. A rolled 13 x 18 cm piece of surgical gauze was then added as a dry support for larval pupation, followed by the addition of adult male and female flies. Each culture flask can support several hundred flies, however, overcrowding of the flask results in a reduced rate of propagation. The cultures were maintained at $25^{o} \pm 0.5^{o}$ in an Hythermco incubator (Pennsauken, N.J.) which was kept at a relative humidity of 80-90% by the addition of several trays of water to the bottom of the incubator. In order to provide appropriate lighting for reproductive purposes, two fluorescent lights inside the incubator were maintained on a twelve-hour light-and-dark cycle. The D. melanogaster cultures grown under these conditions exhibit the standard time-table of development as indicated by Demerec (70).

The large numbers of larvae of similar age used in the various experiments were obtained in the following manner. Two hundred to three hundred pairs of well-fed adult flies were transferred without anesthesia to a 250 ml flask and a powder funnel inserted into a rubber stopper was placed on top. The entire apparatus was inverted onto a 100 x 15 mm plastic Petri dish (Fischer Scientific Co., Fairlawn, N.J.) containing fresh larval culture media. This media was identical to that used for the flies except for the addition of 200 mg penicillin (B grade, Calbiochem, Los Angeles, Calif.) and 50 mg streptomycin sulfate (Nutritional Biochemicals Corp., Cleveland, Ohio) per liter of media.

Egg laying was allowed to proceed for 3-12 hours with the mid-point of the egg-laying period recorded as the time of deposition for the particular dish of eggs. Larval age was calculated assuming the standard 21 hours of embryonic development prior to the hatching of zero-hour larvae and was recorded as the number of hours of development after hatching plus or minus one half of the interval that egg laying was allowed to proceed (for example, 72 $\pm$ 2.5 hour larvae).

The larvae were collected for each experiment by gently rinsing them off of the plate with distilled water into a small beaker. They were rapidly washed with several hundred ml of water by allowing the larvae to settle to the bottom of the beaker and removing the liquid. The larvae were poured gently onto a 15 ml Millipore filter apparatus (Millipore Filter Corp., Bedford, Mass.) on which was placed a very porous polyester material normally used as interfacing in sewing. The larvae were washed with sterile liquid sucrose-salts media (larval culture media made without agar). The polyester material allows the application of gentle suction to remove all excess liquid. The larvae were transferred with a camel's-hair brush to a 100 ml beaker and incubated in a total volume of 0.25 ml. This volume was sufficient to keep the larvae wet without totally immersing them in liquid.

"Rough" treatment of the larvae can result in little incorporation of radioisotopes, and prolonged periods of total submergence in liquid media can injure or kill the larvae. For these reasons, the outlined procedure for handling

the animals was rapid (less than five minutes) and gentle. Larvae isolated in this fashion, incubated for up to eight hours, and then transferred to culture dishes developed at the same rate and reached pupation at the same time as larvae left untouched on their original culture plates.

The animals were normally incubated in 0.20 ml of sterile sucrose-salts media with antibiotics (referred to subsequently as liquid culture media), 25 µl of the specified isotope in water, and 25 µl of distilled water for the control, or 25 µl of polyamine dissolved in water. (For the composition of the incubation media pertaining to each individual investigation, see Results.) Following the addition of incubation media, the beaker was covered with Parafilm which was perforated with small air holes. If the beaker were completely sealed, the concentration of CO_2 would eventually be sufficiently high to anesthetize the larvae. During the incubation period, the larvae were shaken gently in a Dubnoff metabolic shaking incubator (Precision Scientific, Chicago, Ill.) in order to keep them surrounded by the incubation liquid and to prevent them from "wandering" to the walls of the beaker. At the conclusion of incubation, the larvae were thoroughly washed with 100 ml of redistilled water, rapidly weighed on glassine weighing paper, and transferred to a homogenizer.

B. Analysis of Polyamines

The quantitative and qualitative analysis of polyamines used in this work employed a modified fluorescence

procedure previously developed in this laboratory (7,8,24). Ten to 25 mg (wet weight) of washed larvae were homogenized in a one ml glass Duall tissue grinder (Kontes Glass Co., Vineland, N.J.) with 0.4 ml of 0.4 N perchloric acid (PCA). The PCA was diluted with redistilled water from the concentrated 70% reagent (Allied Chemicals, Morristown, N.J.) just prior to use. After vigorous homogenization, the homogenate was centrifuged for ten minutes at 700 x g and 0.2 ml of the supernatent transferred to a 12 ml Bakelite-capped centrifuge tube containing 50 mg of $NaHCO_3$. 0.4 ml of dansyl-chloride reagent (10 mg/ml in acetone, Pierce Chemical Co., Rockford, Ill.) was added, and the dansylation reaction was allowed to proceed with gentle shaking in the dark for sixteen hours. Excess dansyl chloride was reacted with 0.1 ml of an aqueous solution of proline (100 mg/ml) for thirty minutes. Residual acetone was removed *in vacuo* while the tubes were shaken in a water bath. The dansyl amide derivatives of the amines were extracted by agitation on a Vortex-Genie (Scientific Industries, Springfield, Mass.); and following centrifugation, the benzene solution was removed. An aliquot of a standard polyamine solution containing putrescine (P), spermidine (SD), and spermine (SP) in 0.4 N PCA was dansylated and extracted at the same time as the larval homogenate.

The dansyl derivatives were separated via thin-layer chromatography on 20 x 20 cm Silica Gel G plates (*nach* Stahl, Merck AG, Darmstadt, Germany). To prepare the plates, 30 g of silica gel was shaken with 60 ml of redistilled water and

the slurry spread with a Brinkman Desaga variable thickness applicator (Brinkman Instruments, Westbury, N.Y.) set at 200 microns. The plates were allowed to dry overnight and were stored in a desiccator. Immediately prior to use, the plates were activated in an oven for one hour at 105°.

The benzene extracts of the dansylated larval homogenate and polyamine standards were applied to the same plate in no larger than 5 µl aliquots using a 10 µl Hamilton syringe (Hamilton Co., Inc., Whittier, Calif.). Chromatography was performed in ethylacetate:cyclohexane (2:3, v/v). In order to stabilize and intensify the fluorescence following development, the plates were sprayed with approximately 2 g of triethanolamine:isopropanol (1:4, v/v) and dried overnight in a vacuum at 25°.

The fluorescent dansyl spots were quantitated by scanning in a Turner Model 111 flurometer (G. K. Turner Associates, Palo Alto, Calif.) equipped with a TLC scanning attachment and a ten-inch Perkin-Elmer recorder (Hitachi Inc., Japan). The long-wave source (Turner Catalog #110-850) was used with the appropriate filters to allow fluorescence activation at 365 nm and emission measurements above 512 nm.

The areas of the peaks, which were proportional to fluorescence intensity, were determined by planimetry. Standard curves were constructed relating the peak areas of the standard dansylated polyamines to their known concentrations. These curves were linear, passed through the origin, and allowed for the direct determination of the larval polyamine levels.

C. RNA Extraction

RNA was extracted from freshly labeled larvae employing a procedure similar to that used by Greenberg in his work with D. virilis (65). After incubation and washing, the larvae were transferred to a Duall homogenizer in an ice bath. 0.5-1.0 ml of extraction buffer (referred to subsequently as Greenberg buffer) composed of 0.1 M NaCl; 0.01 M sodium acetate, pH 5.1; 1% sequanal grade sodium dodecyl sulfate (SDS) (Pierce Chemical Co.); and 5 µg/ml polyvinyl sulfate (PVS) was added and allowed to remain on ice for fifteen minutes. An equal volume of buffer-saturated distilled phenol was added, and the larvae were homogenized vigorously on ice. Phenol extractions at temperatures higher than 0^{o} were found to lead to substantial degradation of the RNA. 0.5-1.0 ml of cold redistilled $CHCl_3$ was added and mixed intermittently for five minutes on a Vortex-Genie. The $CHCl_3$ prevented the interface formed during centrifugation from sedimenting into the phenol phase and also allowed for a more complete extraction of high molecular weight RNA (71). The homogenate was centrifuged at 500 x g for five minutes at 4^{o}, and the lower phenol-$CHCl_3$ phase removed, The aqueous layer and flocculent interface were extracted twice more with phenol-$CHCl_3$ and, following the final extraction, centrifuged at 1500 x g for ten minutes at 4^{o}. The aqueous layer was removed and extracted with two volumes of anhydrous diethyl ether to remove any residual phenol. Dialysis of the aqueous phase to remove the phenol was found to result in some degradation of

RNA. The ether layer was removed, and the RNA was precipitated by the addition of 2.5 volumes of 80% ethanol containing 0.1 M NaCl; 0.01 M sodium acetate, pH 6, and allowed to stand at -25° for 2-4 hours. In those experiments where polyamine was included in the incubation media, an aliquot of the same polyamine was routinely added to the control (no amine) larvae during RNA extraction.

Glassware used during RNA extraction was thoroughly washed and rinsed in glass-redistilled water. All of the aqueous solutions contacting the RNA during its isolation were either autoclaved or passed through a Millipore filter (HAWP 025). Recovery of purified 16S and 23S E. coli RNA (donated by Dr. Volker Erdmann) extracted using the outlined procedure was 95-100% and possessed the same ratio of 16S:23S as the original RNA preparation.

D. Sucrose-Gradient Analysis of Labeled RNA

The ethanol-precipitated RNA was centrifuged at 1500 x g for ten minutes at 0° and the remaining ethanol removed by inverting the tubes in the refrigerator for five minutes. The RNA was dissolved in 0.3 ml of NET-SDS buffer (72) consisting of 0.1 M NaCl; 1 mM EDTA; 0.01 M Tris-HCl, pH 7.5 (Trizma Brand purchased from Sigma Chemical Co., St. Louis, Mo.); and 0.5% (w/v) SDS. This was layered on linear 5-30% (w/v) sucrose gradients in NET-SDS. The sucrose used was Special Density Gradient Grade from Schwarz/Mann, Orangeburg, N.Y. Centrifugation was performed in a Beckman L2-65B ultracentrifuge (Spinco Division, Palo Alto, Calif.) for

12-13 hours at 27,000 rpm and 22° in the SW 27.1 rotor. Ninety percent of the acid precipitable counts were recovered after centrifugation for thirteen hours at 22° in NET-SDS buffer. This was significantly greater than the recovery obtained using Greenberg's buffer (65) or NET without SDS and centrifuging at 4°.

Fractions were collected from the gradients using an Isco Model D density gradient fractionator and the Model 270 fraction collector. The absorbance at 254 nm was continually monitored by employing the Isco Model UA ultraviolet optical unit and the Model UA-2 ultraviolet analyzer coupled to a ten-inch recorder. (The absorbance of larval RNA at 260 nm is only 3% greater than at 254 nm--see Results.) Reference absorbance readings were made by using the 0.46 and 2.05 optical density unit standards contained in the optical unit. The maximum sensitivity obtainable employing this arrangement was a 0.5 O.D.-unit eliciting a full-scale deflection of the recorder.

The gradients were periodically monitored for linearity by adding blue dextran to the 30% sucrose and determining the linearity of the absorbance tracing during fractionation. The gradients were observed to be linear and reproducible as determined by the absorbance tracing and by the coincidence of the rRNA peaks between gradients in any one experiment. The optical unit has one flow cell which does not compensate for any change in the base-line absorbance contributed by a variable concentration of sucrose in

the 5-30% gradients. However, a blank gradient scanned at maximum sensitivity produced a flat base-line absorbance throughout.

One-ml fractions were collected and acid-precipitated according to Birnboim (73). Two hundred μg of carrier bovine serum albumen (BSA) was added to each fraction followed by the addition of 2.5 ml of cold 10% trichloracetic acid (TCA). The RNA was allowed to precipitate in an ice bath for ten minutes prior to its collection on pre-wetted Whatman GF/A glass-fiber filters (W & R Balston Ltd., England). The filters were rinsed twice with two ml of TCA, air dried for several hours, and then counted in five ml of counting fluid composed of 4 g of Omnifluor (New England Nuclear, Boston, Mass.) per liter of toluene. Control experiments indicated that the GF/A filters were more effective in trapping the TCA-precipitated RNA than either GF/C glass fiber filters or Millipore filters.

The RNA filters were counted in a Nuclear Chicago Mark II Scintillation System. As determined by the channel ratio procedure, the efficiency for counting was approximately 34% with several percent variation between samples.

The area of the abosrbance tracing and of the radioactivity profiles contributed by each RNA species (4S, 18S, and 28S) were determined by planimetry (74) and the specific activities (cpm/O.D. unit) were calculated.

E. Nucleotide Determination

Acid-soluble nucleotides were extracted from larval tissue utilizing a modification of the procedure outlined by Tsuboi and Price (75). 50-100 mg of washed larvae were homogenized in 0.5 ml of 0.5 N PCA at 0^{o}, and the acid-insoluble material was removed by centrifugation at 1500 rpm for ten minutes at 4^{o}. Norit A (Fischer Scientific Co.) was activated before use by washing it in 0.1 N HCl, rinsing several times with redistilled water, and then storing it as the dry powder. Ten mg of the acid-washed Norit was added to the PCA supernatant and the nucleotides allowed to adsorb during continual agitation for thirty minutes. The charcoal was sedimented to the bottom of the tube and washed three times with two ml of redistilled water. The nucleotides were eluted from the Norit by adding 0.5 ml of 0.1 N ammonium hydroxide:50% ethanol (1:1, v/v) and shaking at room temperature for two hours. After centrifugation the supernatant containing the nucleotides was evaporated to dryness under a stream of nitrogen.

The Norit has been shown, under these conditions, to adsorb 99-100% of the acid-soluble nucleotides (75); and in control experiments it was determined that elution of the nucleotides from the charcoal was complete.

The nucleotides were separated using a method of high-voltage paper electrophoresis adapted from Silver et al. (76). The dry nucleotide extract was dissolved in 50 µl of cold electrophoresis buffer (0.015 M trisodium citrate,

0.04% EDTA, pH 4.05) and applied to a 2 x 10 mm blotted-dry area of a 10 x 22 cm piece of buffer-wetted Whatmann #3 MM paper. Electrophoresis was performed at 5-10° in a flat-bed electrophoresis apparatus. The origin was placed at the side of the paper nearest the negative electrode, and electrophoresis was run at 3250 volts for 60-70 minutes at an initial current of 70 ma. (The current normally rose to 90-100 ma after 70 minutes.) Electrophoretic separations employing standard nucleotides indicated that this procedure was sufficient to separate only UTP, the fastest migrating nucleotide, from any of the tri-, di-, or mononucleotides. AMP, UMP, CMP, and GMP could be completely separated from each other when electrophoresis was run for 120 minutes. The nucleotides were visualized by viewing the dried paper with a short-wave ultraviolet light (UVS-12, Ultraviolet Products Inc., San Gabriel, Calif.) and the spots delineated with a pencil. The spots were cut out and shaken in a scintillation vial with 2 ml of water for two hours at 37°. Radioactivity was determined in 15 ml of Aquasol.

F. Determination of DNA Specific Activity

Larvae were incubated with (methyl-^{3}H) thymidine (14.1 mCi/mm, New England Nuclear) in sterile sucrose-salts media according to the procedure previously presented. Following incubation, the larvae were homogenized in phenol-Greenberg buffer employing the same method as that used for RNA extraction. The RNA and DNA thus isolated were separated from each other according to Munro and Fleck (77). 0.15 ml

of freshly prepared 0.3 N NaOH was added to the ethanol-precipitated nucleic acids, and the RNA was hydrolyzed at 37° for 18 hours. The hydrolysate was cooled to 0°, and the DNA was precipitated for 30 minutes by acidifying to a final acid concentration of 0.2 N with PCA. The DNA was washed twice with 0.2 N PCA, dissolved in 1.0 ml of 0.1 N NaOH, and the absorbance at 260 nm determined. One hundred μg of BSA was added as a carrier and the base neutralized with PCA. The degree of ^{3}H-thymidine incorporation was determined by precipitating the DNA with 10% TCA onto Millipore filters and counting in a tolune-phosphor counting fluid. An aliquot of ^{3}H-thymidine (20,000 cpm) added to "cold" larvae during homogenization did not result in any label being deposited on the filter with the DNA. Control experiments revealed that all of the RNA was hydrolyzed by treatment with NaOH while the DNA remained unaffected.

G. Determination of Protein Specific Activity

The larvae were incubated with L-leucine-^{14}C (0.1 mCi/0.059 mg, New England Nuclear) in the liquid culture media, and total protein extracted according to the procedure of Church and Robertson for Drosophila (78). The larvae were homogenized in 5 ml of 2% potassium acetate in 90% ethanol in order to extract any lipids that would prevent some proteins from going into solution. After centrifugation at 2700 x g for five minutes, the pellet was dissolved in 2-3 ml of redistilled water. Four ml of 10% TCA was added and the protein allowed to precipitate at 0° for fifteen minutes. The

precipitate was washed twice with 4 ml aliquots of TCA. Four ml of redistilled water were added to the final protein precipitate and adjusted to pH 10 with NaOH. To remove uric acid (present in 72-hour larvae) and any remaining amino acids from the protein solution, it was dialyzed overnight in the cold against two liters of 0.01 M triethanolamine. The dialysis was sufficient to remove an aliquot of ^{14}C-leucine (20,000 cpm) added in a control experiment during the homogenization of "cold" larvae. Several ml of the dialyzed protein solution was counted in 10 ml of Aquasol and an aliquot was also used for a protein determination.

A microscale Lowry procedure was used for the protein determination (79). 0.2 ml of protein solution in 0.1 N NaOH was added to 1.0 ml of solution A (0.27% sodium potassium tartrate, 0.1% $CuSO_4$, 2% Na_2CO_3) and allowed to incubate for ten minutes at room temperature. 0.2 ml of 1 N phenol reagent (freshly diluted from the commercial 2 N Folin-Ciocalteau reagent, Fischer Scientific Co.) was then rapidly added by means of a blow-out pipette and the color permitted to develop for thirty minutes. Standard solutions of BSA from 10-100 µg were treated in the same manner, and the absorbance at 750 nm was measured in a Beckman DU-2 spectrophotometer.

H. Ornithine Decarboxylase (ODC) Assay

The assay for ornithine decarboxylase (E.C.4.1.1.17) used for Drosophila larvae was a modification of the procedure employed by Wyatt et al. (24) in their studies of the silkmoth, H. cecropia. The larvae of specified ages were

thoroughly washed, weighed, and homogenized in a sufficient volume to yield a 25% (w/v) homogenate. The homogenization media consisted of 10 mM Tris-HCl, pH 7.7; 10 mM EDTA; 5 mM dithiothrietol (A grade, Calbiochem); and 0.25 M sucrose (Schwarz/Mann ultra pure). Both the homogenization and assay media were freshly prepared prior to each assay from frozen concentrated stock solutions. The larvae were vigorously homogenized at 0^{o} in a glass Duall homogenizer and then centrifuged at 2700 x g for fifteen minutes at 4^{o}. One tenth ml of the supernatant was used in each assay. An aliquot was also removed, precipitated with 10% TCA to remove the DTT, and used in a Lowry protein determination. The ODC assay was performed in a total volume of 0.25 ml consisting of 42 mM Tris-HCl, pH 7.1; 0.04 mM pyridoxal-5-P (monohydrate, A grade, Calbiochem); 5 mM DTT; and 1-^{14}C-ornithine (0.2 μCi of DL-^{14}C-ornithine plus sufficient L-ornithine to bring the concentration of the L-isomer to 1 mM). The L-ornithine-HCl, A grade, was purchased from Calbiochem.

The DL-ornithine-1-^{14}C monohydrochloride (29 mCi/mmole, ICN-Tracerlab, Waltham, Mass.) was further purified to lower the counts of the enzyme blank. The isotope solution was made acidic with HCl and stored for 24 hours over solid KOH. The solution was then neutralized and stored at -20^{o} until needed. Isotope treated in this fashion yielded blanks of 40-50 cpm for up to six months, after which time the procedure was repeated.

0.14 ml of the assay mixture was pipetted into 15 ml conical centrifuge tubes in an ice bath. One tenth ml of tissue supernatant was added followed by 10 µl of the ^{14}C-ornithine. The tubes were capped with special rubber stoppers fitted with a plastic center well (Kontes Glass Co.) which contained 0.1 ml of hyamine hydroxide (1 M solution in methanol, ICN). The tubes were incubated for one hour at 37°, and the CO_2 produced was released from solution by the injection of 0.25 ml of 1 N H_2SO_4. The tubes were allowed to remain at room temperature for fifteen minutes, following which the center wells were cut off into scintillation vials containing 10 ml of toluene-phosphor counting fluid. The vials were counted for twenty minutes in a Nuclear Chicago Scintillation Counter with a ^{14}C counting efficiency of 94% (by external standard method). A blank using 0.1 ml of boiled tissue supernatant was carried through the same incubation procedure, and the counts obtained used to correct all assay values. The hyamine hydroxide was found to cause fluorescence for up to sixty minutes. For this reason, the photon monitor on the counter was used at all times.

I. S-Adenosyl Methionine Decarboxylase (SAMD) Assay

S-adenosyl methionine decarboxylase was assayed in a manner similar to that used for ODC. The larvae were homogenized at 0° in a sufficient volume of 100 mM sodium phosphate, pH 7.0; 10 mM EDTA; and 0.25 M sucrose to yield a 25% (w/v) homogenate. Larvae homogenized in Tris-HCl buffer resulted in less SAMD activity than if phosphate buffer were used.

DTT, if employed in either the homogenization or assay media, resulted in a 40% inhibition of enzyme activity.

After centrifugation at 2700 x g for ten minutes at 4^{o}, 0.1 ml of the supernatant was added to 0.145 ml of assay media at 0^{o}. The assay conditions, unless otherwise indicated, were 100 mM sodium phosphate, pH 7; 0.04 mM pyridoxal phosphate; 2.0 mM putrescine; and 0.1 μCi of ^{14}D-S-adenosyl methionine (51.2 mCi/mmole, ICN) with sufficient cold S-adenosyl methionine (Sigma Chemical Co.) added to yield a final substrate level of 0.2 mM. An increased pyridoxal-phosphate concentration of 0.4 mM did not result in any additional activity. Incubation was for sixty minutes at 37^{o}, and the liberated $^{14}CO_2$ was trapped and counted as previously described. Control experiments were performed (see Results) which indicated that these assay conditions yield a maximal SAMD activity proportional to actual enzyme concentration.

J. CsCl Method of RNA Extraction

Washed larvae were homogenized at 0^{o} with a Duall homogenizer in 10-30 volumes of 0.1 M Tris-HCl, pH 8, 4% (w/v) Sarkosyl (sodium lauryl sarcoscine, obtained from Schwarz/Mann). The homogenate was allowed to remain in ice during intermittent homogenization for fifteen minutes when 1.0 gm/ml of solid CsCl (Research Plus Laboratories, Danville, N.J.) was added. The solution was then mixed on a Vortex until all of the CsCl had dissolved. Four ml of the homogenate was then layered onto 1.2 ml of a CsCl cushion (1.707 g/cc, refractive index of 1.4025). The cushion of CsCl had

been previously filtered through a Millipore filter to remove solid impurities. 0.25 ml of the Sarkosyl buffer was layered at the top and the tube was centrifuged in a Beckman SW 50L rotor at 35,000 rpm for twelve hours at 25°. The DNA banding at the interface was removed with a Pasteur pipette. The tube was inverted, and all but the bottom 1 cm sheared off. The purified RNA, which remained as a clear pellet, was then dissolved in buffer and either used immediately or reprecipitated from 2.5 volumes of 80% ethanol, 0.2 M NaCl, and stored at -20°.

K. Polyacrylamide Gel Electrophoresis of RNA

Acrylamide gel electrophoresis was performed according to the procedure outlined by Weinberg et al. (80). Two to three percent agarose gels were made by combining the appropriate amounts of 15% (w/v) acrylamide in water; 2% (w/v) bis-acrylamide in water; N,N,N^1,N^1-tetramethlene diamine; gel buffer (0.04 M Tris-HCl, 0.02 M sodium acetate, 2 mM EDTA, pH 7.4); and solid agarose (Sigma Chemical Co.). The agarose was added to water, autoclaved for fifteen minutes, and added to the remainder of the ingredients warmed to 60°. The polymerization was initiated by the addition of an aliquot of 20% NH_4SO_4, and the gels cast in 0.5 x 7 cm quartz tubes. The acrylamides (Eastman Organic Chemicals, Rochester, N.Y.) were recrystallized according to Loening (81) before use. The gels were allowed to remain in buffer in the cold for at least one week before electrophoresis. Electrophoresis was performed in a twelve-place gel apparatus (Canalco, Rockville,

Md.) coupled to a Beckman Duostat regulated D.C. power supply. The gels were placed in the apparatus with electrophoresis buffer (consisting of gel buffer with 0.5% (w/v) SDS) and pre-run for sixty minutes with fresh buffer in each direction. The RNA (less than 75 µg) was dissolved in 50 µl of SDS-gel buffer with 10% (w/v) sucrose, layered on the gels and electrophoresed at 5 ma/tube for 3-5 hours at 25°.

The gels were scanned at 260 nm in a Joyce-Loebl Chromoscan with the 1 O.D. wedge, 5-077 cam, A_1 of 1010, and F_5 of 0505. They were sliced into 1.5 mm sections, dissolved with 0.1 ml of H_2O_2 (30% solution) at 50°, and counted in 10 ml of Aquasol.

L. Determination of the Amount of ^{14}C-L-Leucine Incorporated into Drosophila Larvae.

Larvae incubated with ^{14}C-L-leucine were washed with 100 ml of water and homogenized in 0.4 ml of freshly prepared 0.05 N sodium bicarbonate, pH 9.5 (82). The homogenate was centrifuged at 2000 x g at 22° for twenty minutes. 0.3 ml of the supernatant was removed and deproteinized by the addition of 0.7 ml of acetone. 50 µl of a 10% dansyl-chloride solution in acetone was added to the extract and allowed to react in the dark for two hours with continuous shaking. The volume of all samples was reduced to 0.5 ml under a stream of nitrogen.

Chromatographic separation of dansyl-L-leucine was accomplished according to Seiler (83). 10-50 µl of solution was spotted on the corner of 20 x 20 cm Silica Gel G plates (see B. Analysis of Polyamines) and chromatography run in

one dimension with benzene-pyridine-acetic acid (16:4:1) for 1.5 hours and in the second dimension with chloroform-benzyl alcohol-acetic acid (70:30:3) for one hour.

The dansyl-L-leucine spot was visualized under ultraviolet light and quantitatively scraped into a scintillation vial. An aliquot of a dansylated L-leucine standard was co-chromatographed with the Drosophila solution to aid in seeing the separated dansyl-L-leucine spot. The radioactivity was determined in 10 ml of toluene-fluor counting fluid containing 4% (w/v) Cab-O-Sil (84).

M. Miscellaneous

The polyamines used in all of the investigations were the HCl-salts purchased from Mann Research Laboratories, New York. (^{3}H)-5-uridine (10.4 Ci/mmole) and L-methionine (methyl-^{14}C) (0.1 mCi/1.1 mg) were obtained from New England Nuclear Corp. Deoxycholic acid was purchased from Sigma Chemical Corp., and Cab-O-Sil was from Cabot Corp., Boston, Mass.

All work concerning the CsCl extraction of RNA was done in conjunction with Dr. Vladimir Glisin who initially developed the method. The sea urchin eggs were collected by Dr. Glisin from Strongylocentrotus purpuratus. The polyribosomal pellet from cultured chick skin fibroblasts was a generous gift of Dr. Helga B. Doty.

RESULTS

A. Stimulation of RNA Synthesis by Spermidine

Due to the indirect evidence relating spermidine to nucleic acid metabolism in D. melanogaster obtained in this laboratory (7,8,35), an attempt was made to determine if exogenously administered spermidine and other amines would have an effect upon RNA synthesis. Such direct evidence has been reported for other systems (10,26-34). A procedure for the labeling of Drosophila larvae by incubation with radioactive precursors in a liquid media (see Materials and Methods) was developed as an alternative to cumbersome microinjection techniques (65).

Initially, it was determined if the endogenous levels of amines in Drosophila larvae could be enriched by incubation with additional amine. Larvae of various ages were incubated with spermidine and putrescine for four hours, following which the larval amine level was determined (Table I). An elevated level of spermidine was observed in 48-95 hour larvae incubated with spermidine, while putrescine remained undetectable. Putrescine is present in low concentrations in Drosophila larvae (7,8), but it is not detectable when only 25 animals are analyzed. Larvae incubated with 10 mM putrescine, however, show a very large increase in their level of putrescine. The spermidine concentrations were virtually unchanged in the presence of exogenous putrescine. 72-hour Drosophila larvae were chosen for further studies due

TABLE I

ENRICHMENT OF DROSOPHILA LARVAE WITH POLYAMINES.

The larvae were incubated for four hours at 25° in a total volume of 0.25 ml of liquid culture media in which the concentration of putrescine (P) or spermidine (SD) was varied. The incubation media contained 0.225 ml S-S media plus 0.025 ml of water (control) or the amine dissolved in water. The animals were washed, homogenized, and analyzed by the fluorescence procedure described in Materials and Methods. (The ages of the larvae are ± 3 hours.)

Amine	Nanomoles polyamine per animal							
	48 hr. larvae		72 hr. larvae		95 hr. larvae		118 hr. larvae	
	SD	P	SD	P	SD	P	SD	P
None	0.64	--	0.34	--	0.32	--	0.33	--
1 mM SD	1.0	--	0.54	--	0.19	--	0.37	--
10 mM SD	0.96	--	0.91	--	0.73	--	0.40	--
1 mM P	0.88	--	0.34	--	0.37	--	0.31	0.18
10 mM P	0.68	0.88	0.26	0.60	--	--	0.33	0.27

to their convenient size and reasonably rapid rate of feeding (the older larvae incorporate substantially less ^{3}H-uridine).

In order to ascertain if the additional amine added to the incubation media was actually entering the cells of the animals, larvae were incubated with ^{3}H-putrescine, and the uptake of putrescine and its subsequent conversion to spermidine monitored for twelve hours (Fig. 1). The amount of labeled putrescine present in the larvae increased rapidly for two hours, where it remained constant until the fourth hour of incubation. The rate of uptake of putrescine again increased and remained approximately linear throughout the remainder of the twelve hours of incubation. There was a slight delay (40 minutes) before tritiated spermidine was detectable, after which the concentration of labeled spermidine rose rapidly for the entire twelve hours of incubation.

The effect of spermidine upon the incorporation of ^{3}H-uridine into RNA of Drosophila larvae is shown in Fig. 2 and Table II. Fig. 2 illustrates the degree of separation of both the absorbance and radioactivity profiles of RNA obtained with the SW 27.1 rotor. Centrifugation at 27,000 rpm for 12-13 hours was sufficient to separate the 28S, 18S, and 4S peaks found in Drosophila RNA. These conditions of sucrose-gradient analysis also were able to distinguish added 16S and 23S E. coli RNA from the 18S and 28S ribosomal RNA species found in Drosophila. The areas of the absorbance and radioactivity profiles were determined planimetrically. This method has been shown to allow for an accurate determination

Figure 1. The rate of uptake of ^{3}H-putrescine and conversion to ^{3}H-spermidine by Drosophila larvae. Several hundred 72 $\pm$ 4 hour larvae were incubated in liquid culture media containing 100 μCi of ^{3}H-putrescine and 1 mM unlabeled putrescine. Thirty animals were removed at time intervals up to twelve hours and perchloric acid extracts prepared. After conversion of the amines in the extract to dansyl derivatives and separation by TLC chromatography (see Materials and Methods), the fluorescent areas were scraped from the TLC plates and extracted with 0.5 ml dioxane. Radioactivity was determined in 10 ml of Aquasol.

FIG. 1

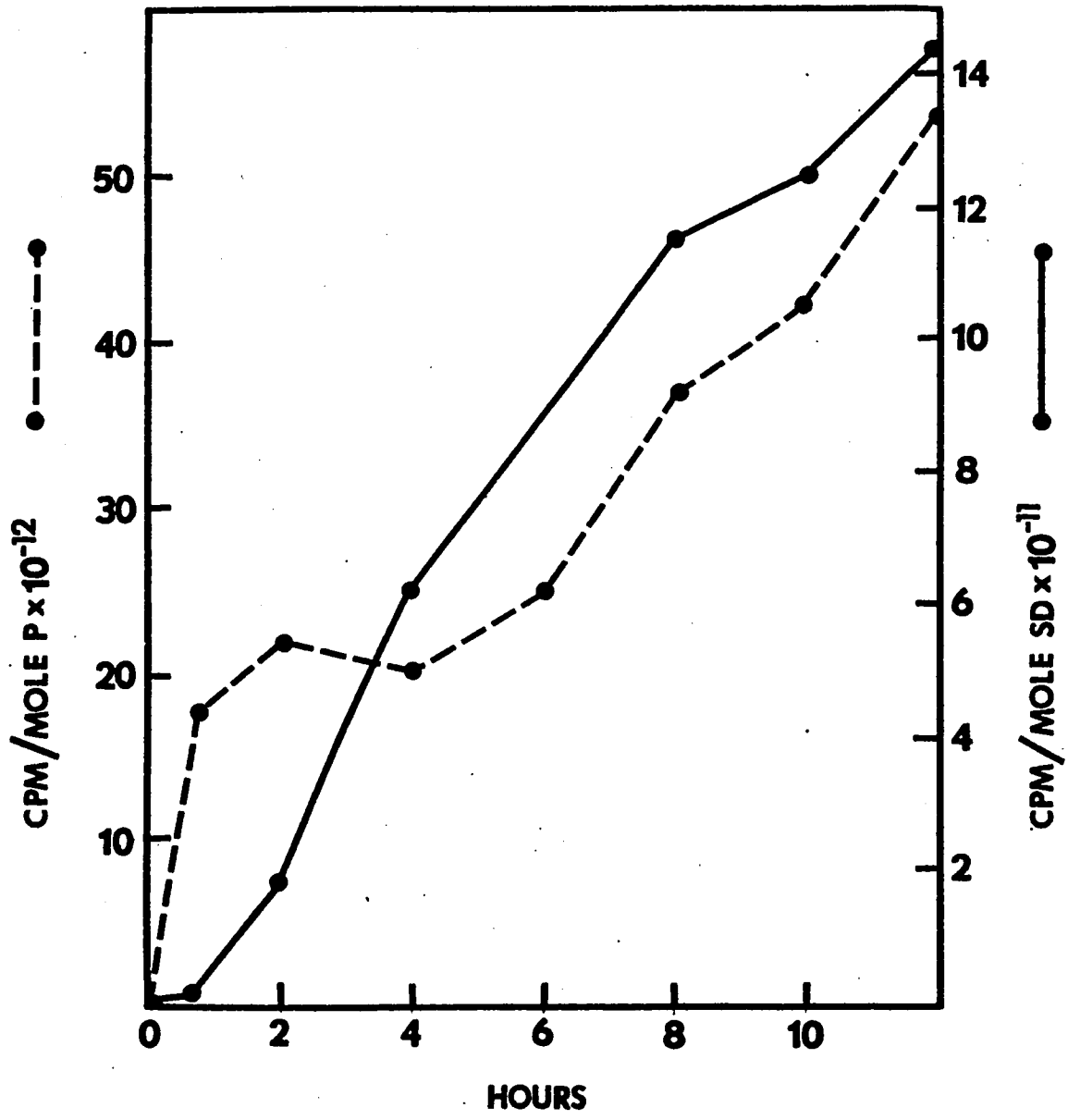

CPM/MOLE P x 10⁻¹²
CPM/MOLE SD x 10⁻¹¹
HOURS
0
2
4
6
8
10
10
20
30
40
50
2
4
6
8
10
12
14

Figure 2. The effect of spermidine upon the incorporation of ^{3}H-uridine into RNA of Drosophila larvae. 72 ± 4 hour Drosophila larvae were pre-incubated for one hour in liquid culture media containing (A) zero and (B) 1 mM spermidine. Incubation was continued for 3.5 hours following the addition of 25 μCi of ^{3}H-uridine. The larvae were washed and the RNA extracted as described in Materials and Methods. The RNA was layered onto 5-30% sucrose gradients in NET-0.5% SDS. Centrifugation was for twelve hours at 27,000 rpm and 22° in the SW 27.1 rotor. One-ml fractions were collected and the absorbance at 254 nm continuously monitored. The RNA was precipitated onto glass-fiber filters and counted in toluene-phosphor counting fluid (see Materials and Methods). Centrifugation is from left to right. (——— = O.D. 254 nm;●———● = cpm.)

FIG. 2

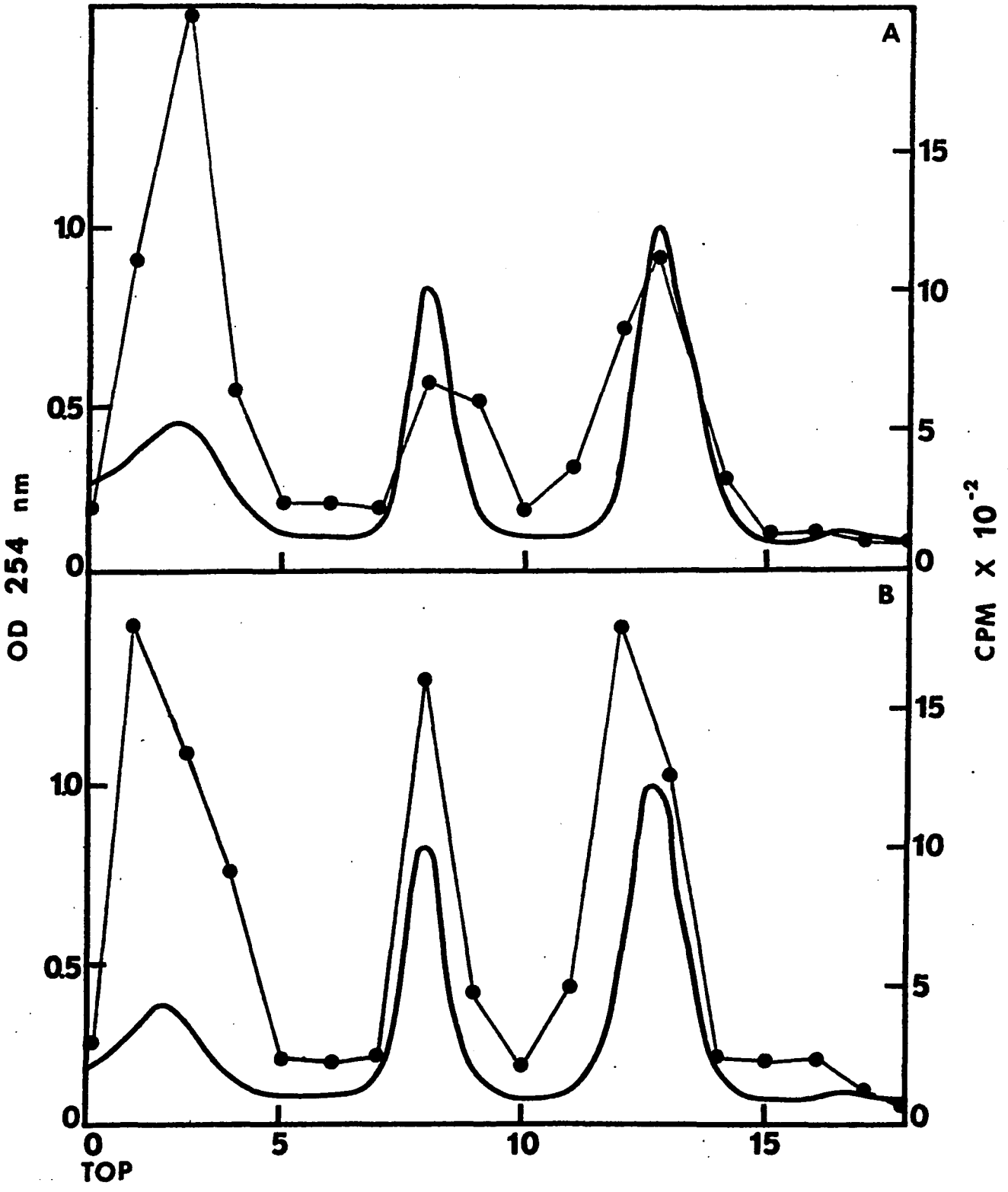


TABLE II

THE EFFECT OF SPERMIDINE ON RNA SYNTHESIS BY DROSOPHILA LARVAE.

RNA was isolated from 72 ± 4 hour Drosophila larvae which were pre-incubated for one hour in 0.25 ml of liquid culture media containing variable concentrations of spermidine followed by incubation in the presence of 20 μCi of ^{3}H-uridine (12 hours), 25 μCi of ^{3}H-uridine (3.5 hours), and 100 μCi of ^{3}H-uridine (20 minutes). The RNA was analyzed by sucrose gradient centrifugation, and the areas of O.D. peaks at 254 nm and of radioactivity profiles on one ml gradient fractions were determined by planimetry. (Specific activity = cpm per 254 O.D. unit for 4S, 18S, and 28S RNA was calculated; relative specific activity to zero spermidine controls = 100 was also determined.

Labeling time	Spermidine (mM)	Specific activity			Relative specific activity		
		4S	18S	28S	4S	18S	28S
20 min.	0	5080	456	452	100	100	100
	1	9350	612	663	190	134	146
3.5 hrs.	0	7200	1392	1400	100	100	100
	1	8560	2256	2280	118	162	162
	5	11430	2487	2672	159	178	190
	10	6680	1896	2112	93	136	150
12 hrs.	0	3396	3707	5504	100	100	100
	1	5430	8455	12000	160	228	215
	5	10170	9682	12242	291	261	220
	120	2220	653	1588	65	17	28

of labeled RNA species by eliminating the contribution of heterogeneous RNA (74,85).

There was significant stimulation of the rate of incorporation of ^{3}H-uridine into the RNA of larvae incubated with 1 and 5 mM spermidine (Table II). The maximum stimulation occurred with 5 mM spermidine following both 3.5 and 12 hours of incorporation time. There was also a definite increase in the relative specific activity of the RNA synthesized in twenty minutes with 1 mM spermidine. The degree of stimulation due to spermidine was observed to increase with the length of labeling and incubation time. The 30-150% rise in RNA specific activity was similar to the extent of amine stimulation observed by most investigators (26,27,30-32) but significantly less than the 300-1200% increase observed in the chick embryo (10,29) and amphibian oocyte (33).

All species of Drosophila larval RNA had increased specific activities due to spermidine. Although many researchers have reported an amine-induced increase in the labeling of total RNA (25,29-31) and rRNA (10,26,28,33), only Wylie (33) has shown an increase in 4S RNA. (The "4S" RNA separated by sucrose-gradient centrifugation and discussed in Tables II-IV is composed of tRNA and 5S rRNA.) Elevated levels of spermidine (120 mM) in the incubation media was observed to be inhibitory to the larvae and caused a decrease, relative to larvae incubated without amine, in the specific activity of the RNA (Table II). High concentrations of amines were actually "toxic" to the larvae resulting in a loss of mobility and/or death.

The specific activity of the 4S RNA was greater than the specific activity of the 18S and 28S rRNA species for labeling times of 20 minutes and 3.5 hours (Table II and Fig. 2). Even though the relative proportion of 4S RNA to 18S and 28S RNA (determined by the absorbance tracing) was in agreement with published observations for Drosophila larvae (65, 78), attempts were made to determine if the relatively large radioactivity peak associated with the 4S RNA was caused by degradation of another RNA species.

The RNA extraction procedure described in Materials and Methods was altered by the addition of two inhibitors of ribonuclease activity--Bentonite (86,87) and diethyl pyrocarbonate (88). There was no apparent difference between RNA extracted with and without Bentonite, and larval RNA extracted in the presence of diethyl pyrocarbonate had relatively more 4S RNA (O.D.) than RNA extracted in the absence of the inhibitor.

In a mixing experiment designed to further examine the effectiveness of the extraction procedure, 72-hour Drosophila larvae were incubated with 25 μCi of ^{3}H-uridine for four hours and the RNA extracted and precipitated in ethanol as described in Materials and Methods. One half of the RNA was stored in ethanol, and the other half was re-extracted with 40 mg of unlabeled larvae. Both RNA samples were centrifuged on separate sucrose gradients and the absorbance and radioactivity profiles determined. 90% of the initially extracted labeled RNA was recovered following re-extraction

with cold larvae, and the distribution of the radioactivity in the two gradients (relative to the 4S, 18S, and 28S absorbance peaks) was the same. Since the specific activity of the 4S peak was not increased subsequent to the second exposure of the RNA to larval nucleases, the radioactivity found with the 4S RNA was assumed not to be due to degradation.

Table III shows the effects of other naturally occurring amines upon the incorporation of ^{3}H-uridine into RNA. Putrescine inhibited the incorporation of label even at the low level of 0.1 mM. This inhibitory effect was perhaps due to the large amount of putrescine (relative to spermidine) taken up by larvae during incubation (Table I). Spermine, which is found in high levels in the chick embryo (10) but is virtually undetectable in Drosophila, caused a small increase in the relative specific activity of the RNA at low concentrations. The higher concentrations were quite inhibitory. The specific activity of RNA from larvae incubated with 0.1-100 mM cadaverine was very similar to RNA from larvae incubated in the absence of the polyamine. Ethylene diamine appeared to inhibit the incorporation of ^{3}H-uridine at almost all of the concentrations studied.

The spermidine stimulation of the specific activity of larval RNA could be explained by reasons other than an increased rate of RNA synthesis. The distribution of label in the $2^{1},3^{1}$-nucleotides of RNA isolated from larvae incubated with and without spermidine was examined in order to determine if the polyamine was causing an increased conversion

TABLE III

THE EFFECT OF RELATED AMINES UPON THE INCORPORATION OF ^{3}H-URIDINE INTO RNA OF DROSOPHILA LARVAE.

72 ± 3 hour Drosophila larvae were pre-incubated for one hour in 0.25 ml of liquid culture media containing the indicated amine at the concentration specified. 25 µCi of ^{3}H-uridine was then added and the larvae incubated for three hours. The larvae were washed, and the RNA was extracted and analyzed on sucrose gradients as described in Materials and Methods and in Table II. Specific activity relative to zero amine controls = 100. (Specific activity = cpm/O.D. 254 nm; P = putrescine; S = spermine; CAD = cadaverine; ED = ethylene diamine.)

	Specific activity			Relative specific activity		
Amine	4S	18S	28S	4S	18S	28S
None	23140	3240	3342	100	100	100
100 mM P	10800	1500	1844	45	46	55
10 mM P	13580	1780	2300	58	55	68
1 mM P	13940	1850	1934	60	57	57
0.1 mM P	17420	2620	3280	75	80	98
None	17490	3936	4708	100	100	100
100 mM S	8930	508	1072	51	13	23
10 mM S	16100	3912	4000	92	71	85
1 mM S	16750	4200	5171	104	106	110
0.1 mM S	19020	4750	5972	118	121	127
None	22160	3120	3272	100	100	100
100 mM CAD	22820	2780	3178	103	89	97
10 mM CAD	20370	2932	2879	92	94	88
1 mM CAD	21560	3307	3236	97	106	99
0.1 mM CAD	21970	3235	3431	99	103	105
None	23580	2520	2440	100	100	100
100 mM ED	17490	1030	1164	74	41	47
10 mM ED	18780	2190	2464	79	87	100
1 mM ED	17500	1908	1832	74	75	75
0.1 mM ED	22042	2740	2462	93	108	100

of ^{3}H-uridine into nucleotide triphosphates other than UTP (Table IV). 90-95% of the radioactivity present in the RNA was found, following alkaline hydrolysis, in UMP and CMP. Although most of the label was in UMP, a significant amount (20-24%) was present as CMP in the hydrolyzed 18S and 28S RNA. This was in contradiction to Rubenstein and Clever (89) who reported no detectable conversion of ^{3}H-uridine into cytosine nucleotides in RNA from cultured salivary glands of C. tentans. Plagemann (90), however, observed that the relative proportion of uridine incorporated into CMP of RNA from rat hepatoma cells increased to a level equal to the cytosine concentration in the RNA after eight hours of incubation. Such differences are most likely due to variations in culture conditions and ability of the cells to metabolize exogenously administered uridine.

The relative proportion of CMP was significantly higher in the 4S RNA (31-34%). This increase can be partially explained by the greater proportion of cytosine (+8%) found in 4S Drosophila RNA relative to 18S and 28S RNA (91). The distribution of label found in all of the species of RNA was the same in larvae incubated with and without spermidine.

Spermidine and the related amines could also be affecting the uptake of ^{3}H-uridine and its conversion to UTP. Table V shows the effect of the amines of the ^{3}H-UTP pool. (Because of the large amounts of material required to obtain pool data related to the actual concentration of the specific

TABLE IV

LABELED NUCLEOTIDES FROM RNA OF DROSOPHILA LARVAE INCUBATED WITH AND WITHOUT SPERMIDINE.

75 mg of 72 ± 4 hour Drosophila larvae were incubated in 0.25 ml of liquid culture media for 6.5 hours with 50 μCi of ^{3}H-uridine and zero (control) or 1 mM spermidine. The RNA was extracted (see Materials and Methods) and separated on 5-30% sucrose gradients in NET-0.5% SDS centrifuged for 13 hours at 27,000 rpm and 22°. The individual RNA species (4S, 18S, and 28S) were collected and precipitated from ethanol following the addition of 200 μg of E. coli 4S RNA. The RNA was hydrolyzed in 0.3 N NaOH for 12 hours at 37° and the nucleotides separated via high-voltage electrophoresis. The radioactivity was determined by counting the eluted nucleotides in Aquasol. 90-95% of the radioactivity was present in CMP and UMP. (SD = spermidine.)

	CMP (cpm)	Percent control	UMP (cpm)	Percent control	CMP + UMP	Percent control	UMP/ CMP
Control							
4S	1800	100	3830	100	5630	100	2.12
18S	3240	100	10744	100	13984	100	3.31
28S	5354	100	18005	100	23359	100	3.36
1 mM SD							
4S	3190	177	6033	157	9223	164	1.89
18S	4846	149	17730	165	22576	161	3.66
28S	7870	147	27092	150	34962	149	3.45

TABLE V

EFFECT OF AMINES ON THE INCORPORATION OF ^{3}H-URIDINE INTO UTP.

60-90 mg (wet weight) of 72 ± 6 hour Drosophila larvae were incubated for three hours in 0.25 ml of liquid culture media containing 25 µCi of ^{3}H-uridine and 1 mM of the indicated amine. Free nucleotides were extracted with 0.5 ml of 0.5 N PCA and adsorbed onto 10 mg of Norit as described in Materials and Methods. The dried nucleotides were dissolved in 50 µl of electrophoresis buffer (0.015 trisodium citrate, pH 4.05; 0.04% EDTA) containing 0.1 mg of UTP and electrophoresed on Whatmann 3MM at 3250 V for 60-65 minutes. The UTP spot was visualized under ultraviolet light, placed in a scintillation vial with 2.0 ml of water, and shaken at 37° for three hours. Radioactivity was determined in 15 ml of Aquasol. SD = spermidine; P = putrescine; S = spermine; CAD = cadaverine; ED = ethylene diamine. Specific activity = cpm/gm larvae; the "relative specific activity" to the zero amine controls = 100 was determined.

Amine	Specific activity	Relative specific activity
None	4451	100
1 mM SD	4418	99
1 mM P	4796	107
1 mM S	3992	89
1 mM CAD	4125	92
1 mM ED	4411	99

triphosphates (92,93), the data presented is in terms of gram wet weight of larvae.) None of the amines investigated altered substantially the specific activity of UTP relative to the control (larvae incubated without amine).

The effect of spermidine on the turnover of methyl-labeled Drosophila RNA was determined as shown in Table VI. Larvae were incubated with ($^{14}CH_3$)-L-methionine instead of ^{3}H-uridine due to the observation made by several authors (94,95) that nucleotides released from RNA by turnover appear to be preferentially re-incorporated into newly synthesized RNA.

The Drosophila larvae were incubated for 3.5 hours with labeled methionine and then "chased" for three and six hours in the presence of cold methionine with and without added spermidine. The relative specific activities of the RNA species are similar to those reported by Greenberg (65) in methyl-labeled RNA from D. virilis larvae. There was very little turnover of any of the species of RNA after three hours and a slight increase of specific activity in some species after six hours. The specific activities of the RNA's of larvae incubated in the presence and absence of 1 mM spermidine were similar after both three and six hours.

There are no reported values for the half-lives of Drosophila larval RNA in the literature. Price (96), however, has observed that RNA from ribosomes in larvae of the blowfly, C. erythrocephala, was not degraded but stored for subsequent needs during pupation (see Discussion).

TABLE VI

TURNOVER OF ^{14}C-METHYL-LABELED DROSOPHILA RNA.

72 ± 6 hour Drosophila larvae were incubated with 5 μCi of ($^{14}CH_3$)-L-methionine in 0.25 ml of culture media for 3.5 hours. The larvae were washed thoroughly and placed into beakers containing fresh liquid culture media with 1 mM cold L-methionine and allowed to stand for sixty minutes. An aliquot of spermidine or water was added to yield the indicated amine concentration and the larvae were incubated for the specified time. The larvae were thoroughly washed and the RNA extracted (see Materials and Methods). The purified RNA was separated on 5-30% sucrose gradients in NET-0.5% SDS and 1.0 ml fractions were collected. The radioactivity was counted following acid precipitation onto filters, and the specific activity (cpm/O.D. 254 nm) was obtained by relating the areas of the O.D. peaks to the corresponding counts. (See Materials and Methods and Table II for details.) (SD = spermidine.)

		Specific activity		
Amine	Time	4S	18S	28S
---	0	2200	524	429
0	3 hrs.	2420	552	496
1 mM SD	3 hrs.	2550	468	510
0	3 hrs.	1920	452	416
1 mM SD	3 hrs.	1895	470	425
0	6 hrs.	2290	594	586
1 mM SD	6 hrs.	2640	584	600
0	6 hrs.	2040	614	570
1 mM SD	6 hrs.	2350	602	506

Caldarera (10) has shown that polyamines cause increased incorporation of ^{3}H-formate into DNA of the chick embryo. Drosophila larvae were incubated with ^{3}H-thymidine in the presence and absence of spermidine (Table VII). The specific activity of DNA from larvae incubated three hours with spermidine was slightly lower than in the animals incubated without the polyamine. The relative specific activities of the DNA were similar after eight and twelve hours of incubation. Table VIII shows that the slight decrease in the labeling of DNA after three hours in the presence of spermidine was probably due to the lower specific activity of the TTP pool.

Goldstein (26) has observed that added polyamine inhibited protein synthesis in Walker 256 carcinoma cells. The effect of spermidine upon the incorporation of ^{14}C-L-leucine into total protein of Drosophila larvae is shown in Table IX. Larvae were incubated with and without polyamine for 3-12 hours and the specific activity of the total protein monitored throughout. The amount of label in the protein increased during the twelve hours of incubation. One mM spermidine caused a slight increase in the specific activity of protein relative to the zero amine controls after three and eight hours of incubation.

In order to determine if these increases were due to an increased uptake of ^{14}C-L-leucine from the culture media, the effect of spermidine upon the leucine pool was investigated as described in Table X. The specific activity of the

TABLE VII

THE EFFECT OF SPERMIDINE UPON THE INCORPORATION OF ^{3}H-THYMIDINE INTO DNA IN DROSOPHILA LARVAE.

72 ± 8 hour larvae were incubated in liquid culture media containing zero (control) or 1 mM spermidine and 25 µCi ^{3}H-thymidine for the indicated times. DNA and RNA were extracted, the RNA was hydrolyzed in NaOH, and the DNA was acid precipitated according to Materials and Methods. The absorbance of the DNA at 260 nm was recorded and the radioactivity determined by counting in Aquasol. (Specific activity = cpm/O.D. 260 nm; SD = spermidine.)

Amine	Incubation time	Specific activity	Percent control
Control	3 hrs.	9610	100
1 mM SD	3 hrs.	9150	95
Control	3 hrs.	6830	100
1 mM SD	3 hrs.	5700	88
Control	8 hrs.	64600	100
1 mM SD	8 hrs.	66500	102
Control	12 hrs.	120100	100
1 mM SD	12 hrs.	125500	105

TABLE VIII

THE EFFECT OF SPERMIDINE UPON THE INCORPORATION OF ^{3}H-THYMIDINE INTO TTP.

30-90 mg (wet weight) of 72 ± 6 hour Drosophila larvae were incubated in liquid culture media containing zero (control) or 1 mM spermidine and 25 µCi of ^{3}H-thymidine for the indicated times. Acid-soluble nucleotides were extracted and separated by high-voltage electrophoresis as described in Table V. Due to the unavailability of TTP as a standard, the area between the fastest migrating nucleotide (UTP) and the slowest moving triphosphate (ATP) was cut into 2 x 3 cm sections, the radioactivity eluted with 2.0 ml of water, and the radioactivity determined in Aquasol. After three hours of incubation, 75-80% of the label was found in the two sections nearest the UTP spot. This was assumed to be TTP. (UTP had only 2% of the counts and no nucleotide mono- or di-phosphates migrate between ATP and UTP.) Specific activity relative to the zero amine control = 100. (Specific activity = cpm/gm larvae; SD = spermidine.)

Amine	Incubation time	Specific activity	Percent control
Control	3 hrs.	13804	100
1 mM SD	3 hrs.	12625	91
Control	8 hrs.	8575	100
1 mM SD	8 hrs.	9357	109

TABLE IX

THE EFFECT OF SPERMIDINE UPON THE INCORPORATION OF ^{14}C-L-LEUCINE INTO TOTAL PROTEIN IN DROSOPHILA LARVAE.

72 ± 6 hour larvae were incubated in liquid culture media containing zero (control), 1 mM, or 10 mM spermidine and 10 µCi ^{14}C-L-leucine for the indicated times. Total protein was extracted as described in Materials and Methods. Protein concentration was determined using a microscale Lowry procedure and the radioactivity obtained by counting in Aquasol. (Specific activity = cpm/ug protein; SD = spermidine.)

Amine	Incubation time	Specific activity	Percent control
Control	3 hrs.	14.8	100
1 mM SD	3 hrs.	16.5	111
10 mM SD	3 hrs.	14.1	95
Control	5 hrs.	30.8	100
1 mM SD	5 hrs.	30.9	100
Control	8 hrs.	71.4	100
1 mM SD	8 hrs.	76.5	107
Control	12 hrs.	82.5	100
1 mM SD	12 hrs.	89.5	109

TABLE X

THE EFFECT OF SPERMIDINE UPON THE INCORPORATION OF ^{14}C-L-LEUCINE INTO DROSOPHILA LARVAE.

60-80 mg (wet weight) of 72 ± 6 hour Drosophila larvae were incubated in 0.25 ml liquid culture media containing zero (control) or 1 mM spermidine and 10 µCi of ^{14}C-L-leucine for the indicated times. The total amino acids were extracted in sodium bicarbonate, pH 9.05, deproteinized, and dansylated as described in Materials and Methods. 30 µl of the dansylated larval extract and 10 µl of a dansylated leucine standard were spotted onto 20 x 20 cm Silica Gel G plates and chromatography run in two dimensions. The dansyl-L-leucine spot was visualized under ultraviolet light and quantitatively scraped into a scintillation vial. Radioactivity was determined in 10 ml of toluene-fluor counting fluid containing 4% Cab-O-Sil. Specific activity = cpm/gm larvae; SD = spermidine.

Amine	Incubation time	Specific activity	Percent control
Control	3 hrs.	8044	100
1 mM SD	3 hrs.	10059	125
Control	8 hrs.	8911	100
1 mM SD	8 hrs.	7890	88

^{14}C-L-leucine pool was saturated by three hours of incubation. There was a 25% relative increase in the specific activity of leucine from larvae incubated with spermidine by three hours. This increase was probably the reason for the rise (+11%) in protein specific activity seen at three hours (Table IX). However, after eight hours, there was a relative decrease (-12%) in the leucine pool due to spermidine. This would increase to 21% the small relative rise in the specific activity of total larval protein observed after eight hours (Table IX).

B. Ornithine Decarbosylase and S-Adenosyl-L-Methionine Decarboxylase in Drosophila Larvae

Although polyamine levels have been studied in several insect tissues (8,9,24,35), their biosynthetic enzymes in these tissues have been investigated in only one instance (ODC activity has been observed in Cecropia) (24). An attempt was made to determine if there were ODC and SAMD activity present in Drosophila larvae, to develop a reliable set of assay conditions for these enzymes, and to investigate any variation in ODC and SAMD activity during larval development.

Drosophila larvae were assayed for ODC activity as described in Materials and Methods. Significant levels of ODC activity were obtained from the larvae using assay conditions adapted from Wyatt et al. (24). The addition of 5 mM DTT to the assay and homogenization media resulted in 2-3 times the ODC activity obtained in the absence of DTT. Similar observations have been made by other authors (24,46).

As in other tissues (46,48), Drosophila ornithine decarboxylase seems to have a requirement for pyridoxal-phosphate. Without pyridoxal-phosphate in the assay media, very little ODC activity was detected. The use of pyridoxal-phosphate stock solutions stored (at -20°) for more than several weeks also yielded low ODC activity.

In order to determine if there were any enzyme activity remaining in the pelleted larval tissue following the 2700 x g spin (see Materials and Methods), the pellet was washed twice with homogenization media and sequentially extracted with 0.1 and 1.0% deoxycholate. Neither extract assayed in the normal manner (Materials and Methods) was found to possess ODC activity above the blank (boiled tissue) level.

The variation in ODC activity with increasing L-ornithine concentration was determined with a tissue homogenate from 72-hour Drosophila larvae (Fig. 3). An L-ornithine concentration of 1 mM was found to give near saturation of the enzyme present and was used in all subsequent assays. Through the use of the double-reciprocal plot, the apparent K_m for ODC was determined to be 0.27 mM, which was close to the K_m of 0.2 mM found in Cecropia (24).

The variation in ODC activity with increasing concentration of enzyme was determined with an homogenate from 48-hour larvae (Fig. 4). The amount of larval homogenate in the total assay volume of 0.25 ml was varied from 25-100 µl. ODC activity was found to be proportional to increasing

Figure 3. Effect of increasing concentration of L-ornithine on ODC activity. Each assay contained 0.1 ml supernatant from homogenates of 72 ± 3 hour *Drosophila* larvae (see *Materials and Methods*). The ODC assays were performed in a total volume of 0.25 ml consisting of 42 mM Tris-HCl, pH 7.1; 0.04 mM pyridoxal-phosphate; and 5 mM DTT. The concentration of L-ornithine was varied from 0.1-1.0 mM while the specific activity was maintained at 1.0 mM L-ornithine/0.5 μCi DL-^{14}C-ornithine.

FIG. 3

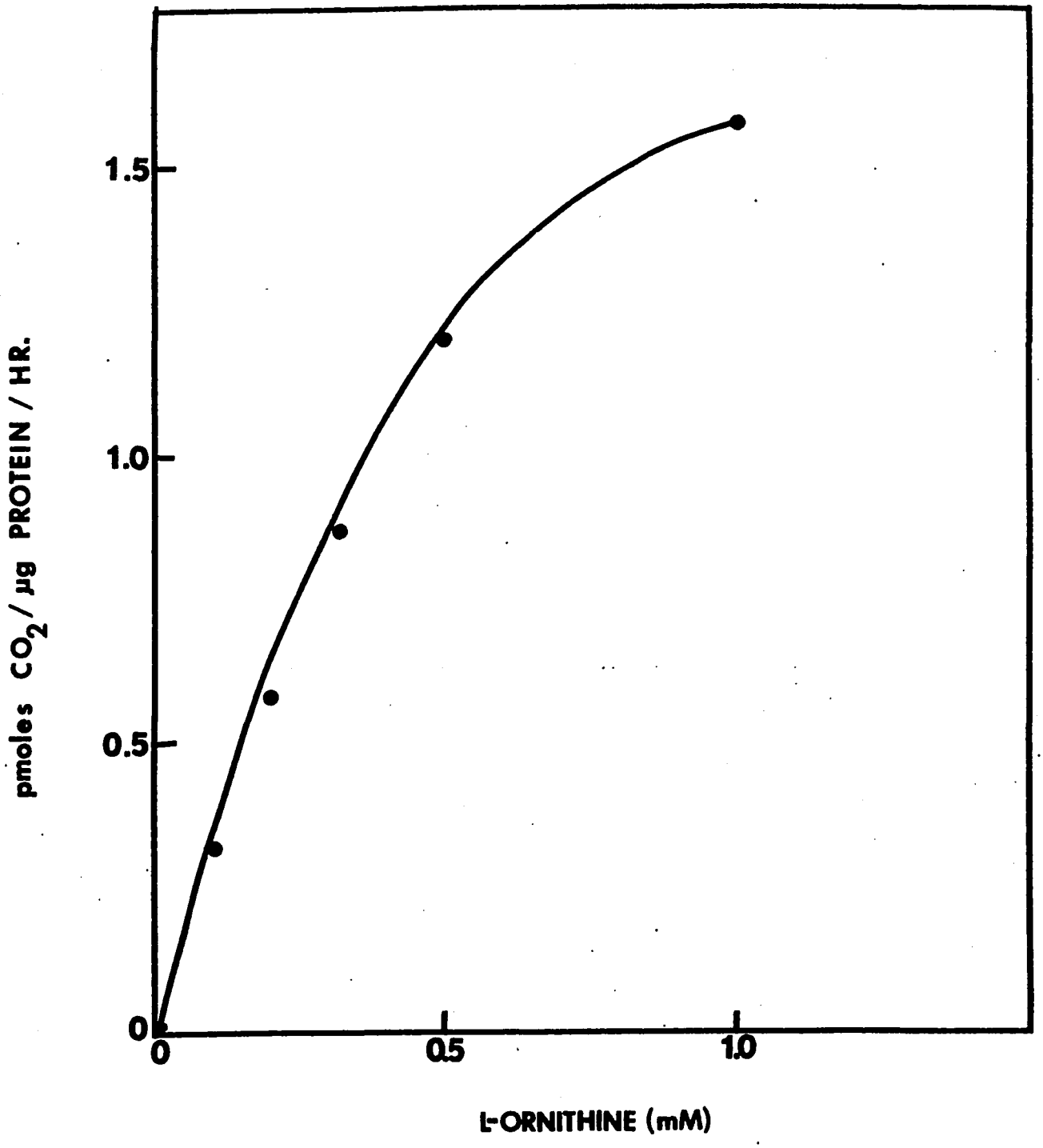
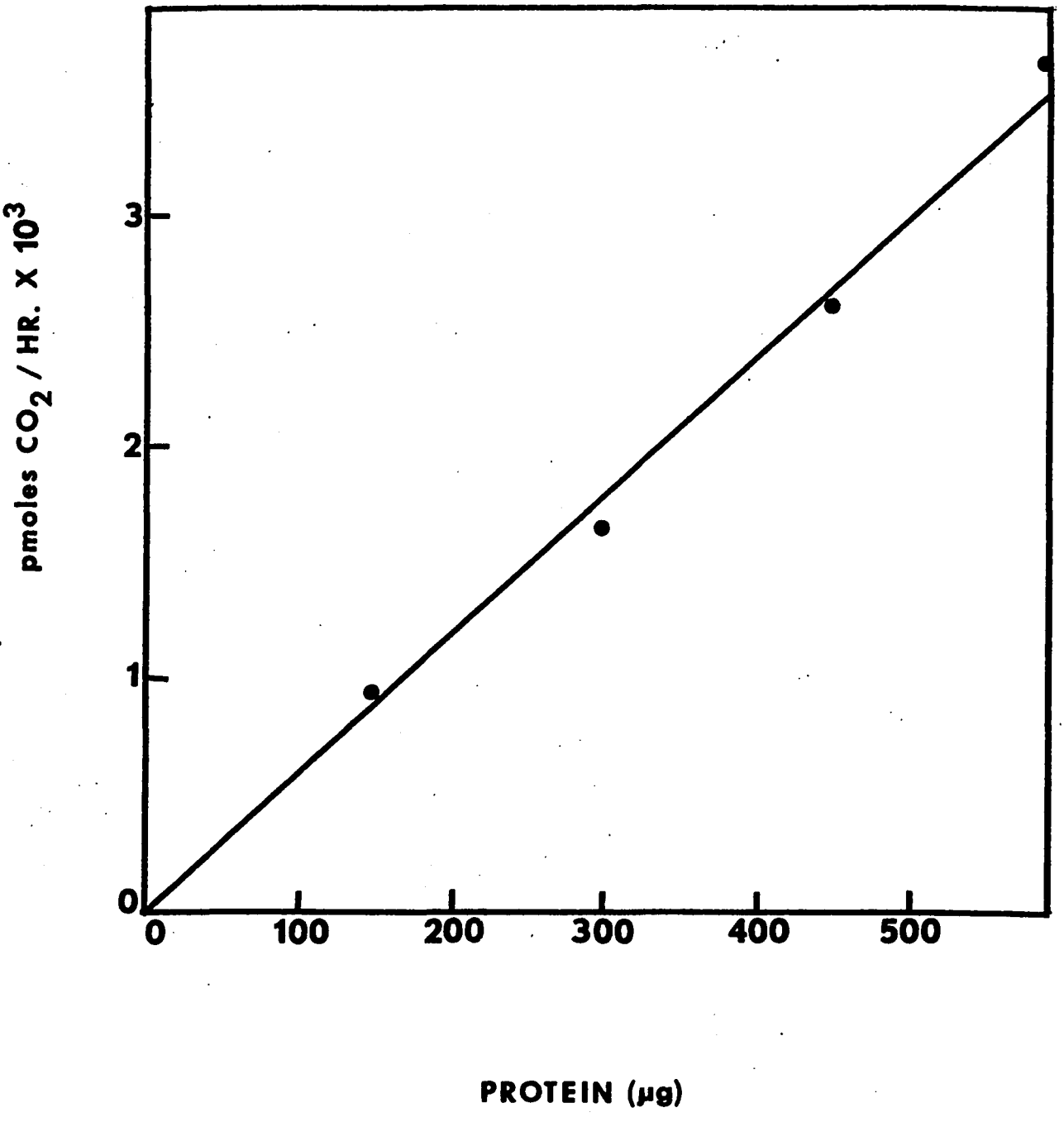


Figure 4. Variation in ODC activity with increasing concentration of enzyme. The volume of supernatant from an homogenate of 48 ± 4 hour Drosophila larvae in each assay was varied from 25-100 µl. ODC activity was determined in a total volume of 0.25 ml in 42 mM Tris-HCl, pH 7.1; 0.04 mM pyridoxal-phosphate; 5 mM DTT; 0.2 µCi of DL-^{14}C-ornithine; and a total L-ornithine concentration of 1 mM. The protein concentration in each aliquot of supernatant was determined as described in Materials and Methods.

FIG. 4



enzyme concentration (increasing concentration of homogenate) up to 5-6 pmoles CO_2/µg protein/hour corresponding to 100 µl of homogenate. Figure 5 shows that the release of CO_2 under the assay conditions used was linear throughout the sixty minutes incubation time.

Kay et al. (50) indicated that an increased concentration of amino acids (+7 mM) in the culture media of lymphocytes caused a stimulation in ODC activity. A similar rise in liver ODC activity has been seen in rats fed casein extract (97). 72-hour Drosophila larvae were incubated in 0.25 ml of liquid culture media containing 0.2 and 2% casein extract (approximately 2.4 and 24 mM in amino acids) for four hours. The larvae were washed, homogenized, and assayed for ODC activity as described in Materials and Methods. No increase in ODC activity above control levels (larvae incubated without amino acids) was observed.

S-adenosyl-L-methionine decarboxylase was assayed in Drosophila larvae in a manner similar to ODC. Phosphate buffer was, however, used during homogenization and assay because Tris-HCl was found to inhibit by 30-40% SAMD activity. This has also been observed with SAMD from the rat prostate (98).

The variation in SAMD activity from 72-hour Drosophila larvae with an increasing concentration of S-adenosyl-L-methionine is shown in Fig. 6. The saturated level of substrate was 0.2 mM, and this concentration was used in all succeeding assays. The apparent K_m (determined by a double-reciprocal plot) was 0.035 mM. This compares very well with

Figure 5. Effect of increasing incubation time on ODC activity. Each assay contained 0.1 ml supernatant from an homogenate of 72 ± 3 hour Drosophila larvae (see Materials and Methods). The ODC assays were performed in a total volume of 0.25 ml consisting of 42 mM Tris-HCl, pH 7.1; 0.04 mM pyridoxal-phosphate; 5 mM DTT; 0.2 μCi DL- ^{14}D-ornithine; and a total L-ornithine concentration of 1 mM. The decarboxylation reaction was allowed to proceed at 37° for 15, 30, 45, and 60 minutes before the addition of 1 N H_2SO_4.

FIG. 5

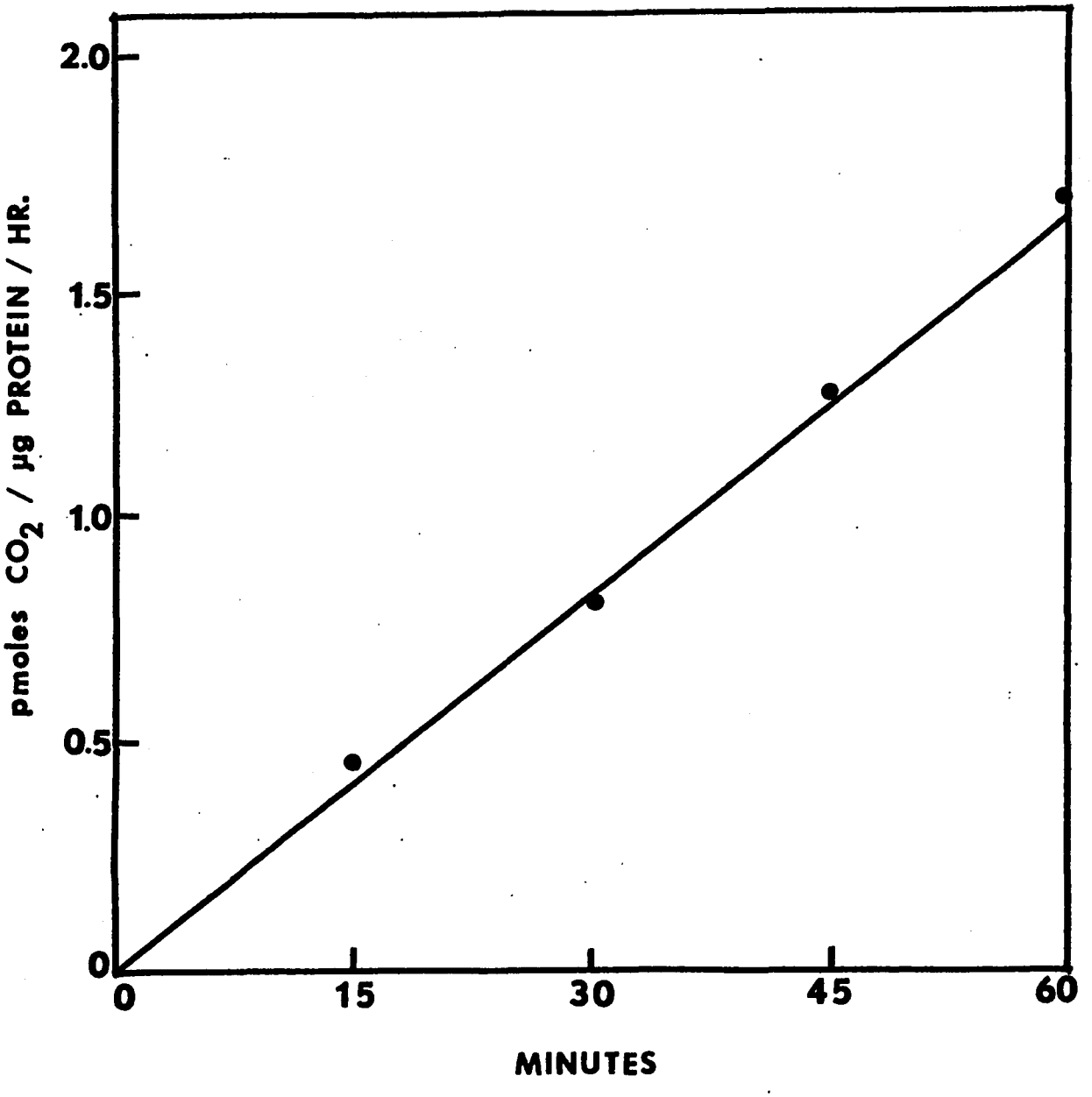
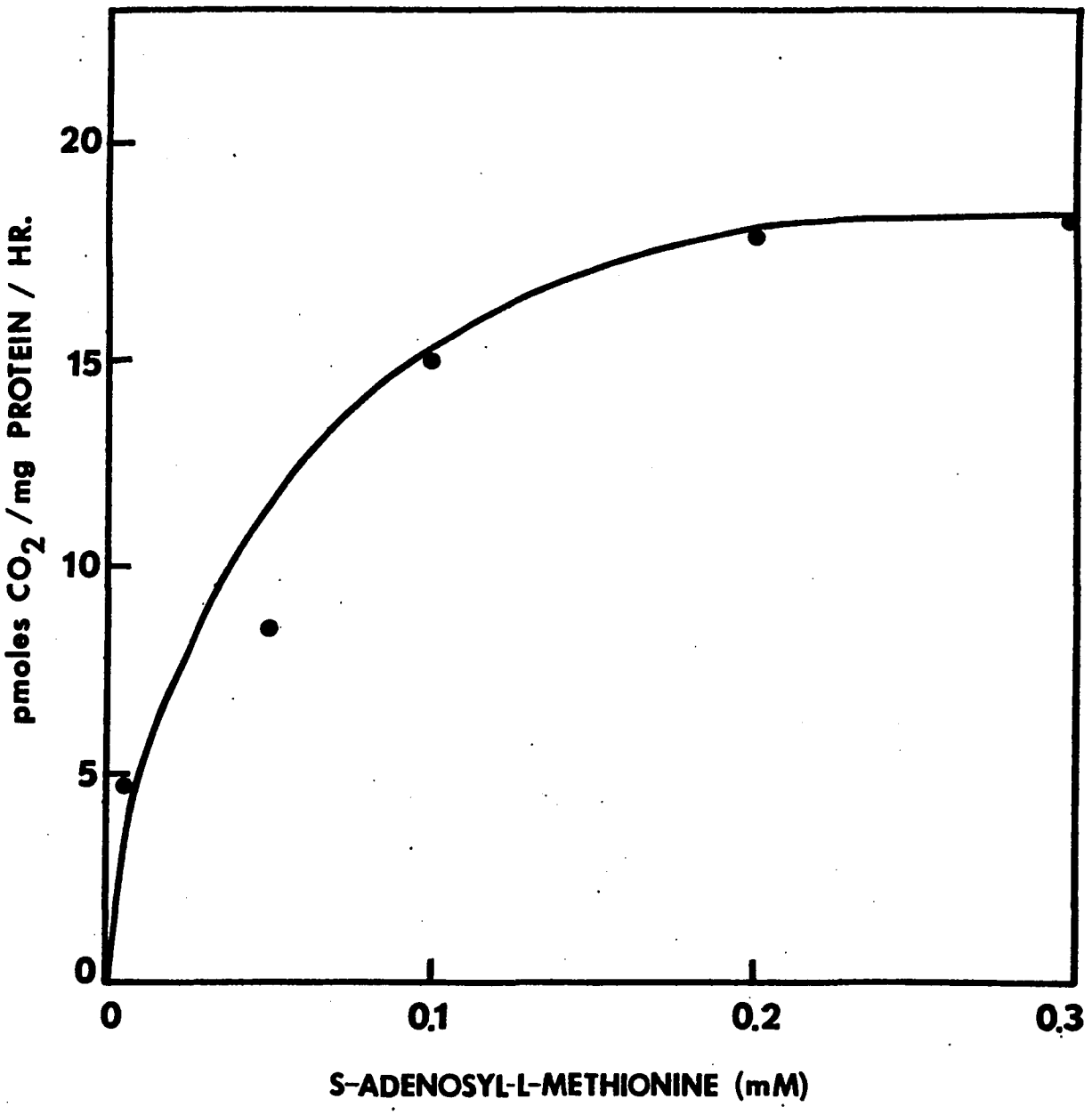


Figure 6. Effect of increasing concentration of S-adenosyl-L-methionine on SAMD activity. Each assay contained 0.1 ml of supernatant from an homogenate of 72 ± 6 hour *Drosophila* larvae (see Materials and Methods). The SAMD assays were performed in a total volume of 0.25 ml consisting of 100 mM sodium-phosphate, pH 7.0; 0.04 mM pyridoxal-phosphate; and 0.1 μCi of ^{14}C-S-adenosyl-L-methionine. The total concentration of S-adenosyl-L-methionine was varied up to 0.3 mM by the addition of cold substrate.

FIG. 6


20
15
10
5
0
pmoles CO_2 /mg PROTEIN / HR.
0
0.1
0.2
0.3
S-ADENOSYL-L-METHIONINE (mM)

the K_m values of 0.036 mM for rat liver (55), 0.05 mM for rat prostate (98), and 0.052 mM for rat brain (57).

Putrescine has been shown to stimulate SAMD activity from nearly all of the tissues that have been studied (51-55, 99). The stimulation of SAMD activity from 40-hour Drosophila larvae by varying concentration of putrescine is shown in Fig. 7. 2.0 mM putrescine was found to stimulate SAMD activity by 15-20 times the activity assayed without additional putrescine. Putrescine concentrations of up to 8.0 mM did not yield any greater increase in SAMD activity above that obtained with 0.25 mM. The apparent K_m for putrescine was determined to be 0.25 mM, which compares with the K_m value of 0.33 mM calculated for rat liver (55).

The linearity of the assay for SAMD activity with respect to the concentration of the enzyme (homogenate) and the length of incubation time at 37^{o} is shown in Figures 8 and 9. The assays shown were performed in the absence of additional putrescine. (SAMD activity was also proportional to the amount of homogenate and incubation time in the presence of 2.0 mM putrescine.) Pegg and Williams-Ashman (98), however, reported that partially purified SAMD from the rat prostate, assayed in the absence of putrescine, decarboxylated ^{14}C-SAM for only the first fifteen minutes of the incubation time.

Drosophila larvae from 24-96 hours old were assayed for ODC and SAMD activity using the conditions outlined in Materials and Methods and shown to yield activities

Figure 7. Putrescine stimulation of SAMD activity. Each assay contained 0.1 ml of supernatant from an homogenate of 40 ± 3 hour Drosophila larvae (see Materials and Methods). The SAMD assays were performed in a total volume of 0.25 ml consisting of 100 mM sodium-phosphate, pH 7.0; 0.04 mM pyridoxal-phosphate; 0.05 μCi ^{14}C-S-adenosyl-L-methionine; and a total S-adenosyl-L-methionine concentration of 0.2 mM. 0-2.0 mM putrescine was added to each assay mixture.

FIG. 7

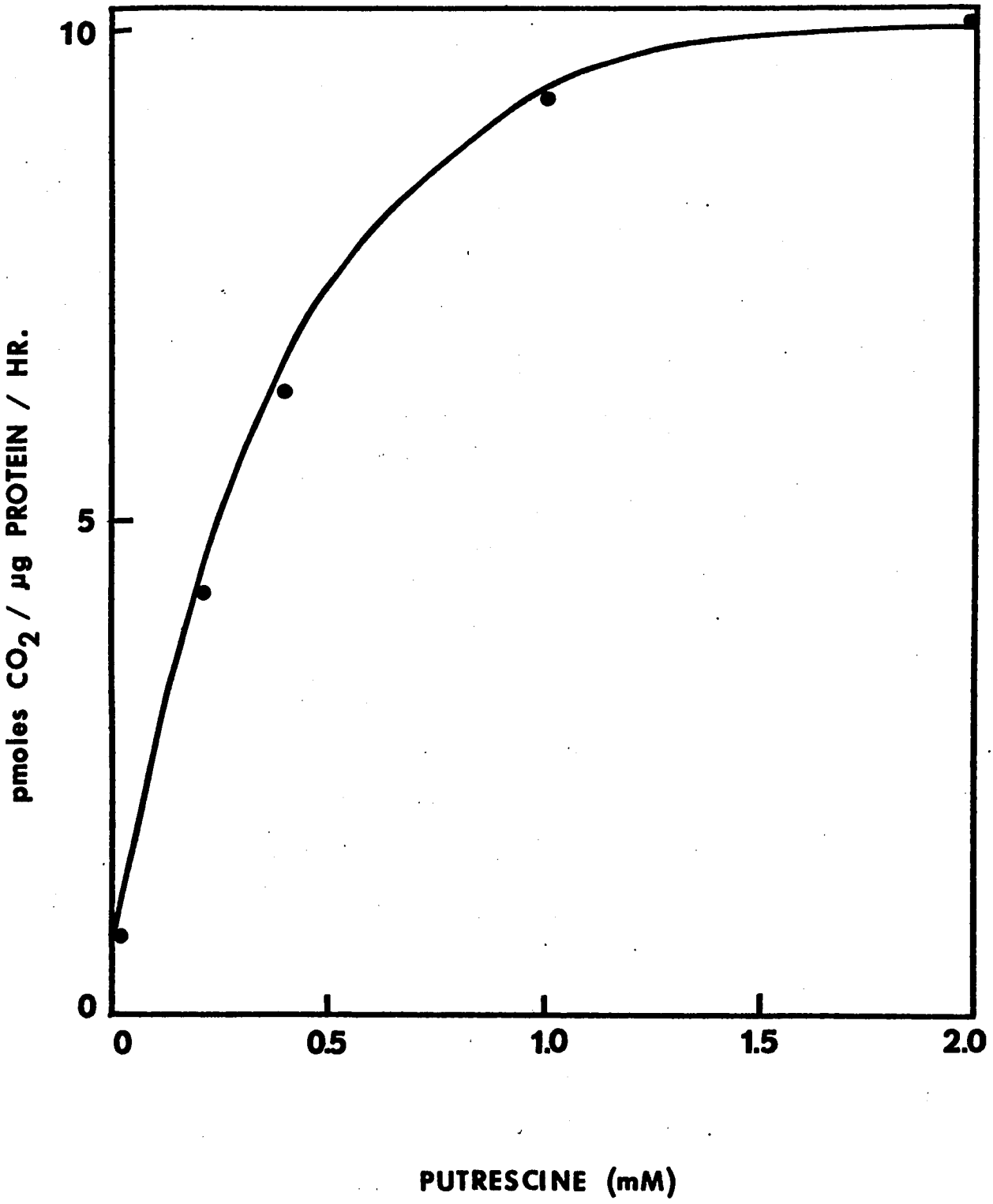


Figure 8. Variation in SAMD activity with increasing concentration of enzyme. The volume of supernatant from an homogenate of 24 ± 5 hour Drosophila larvae was varied from 25-100 µl. SAMD activity was determined in a total volume of 0.25 ml consisting of 100 mM sodium phosphate, pH 7.0; 0.04 mM pyridoxal-phosphate; 0.1 uCi ^{14}C-S-adenosyl-L-methionine; and a total S-adenosyl-L-methionine concentration of 0.20 mM. The protein concentration in each aliquot of supernatant was determined as described in Materials and Methods.

FIG. 8

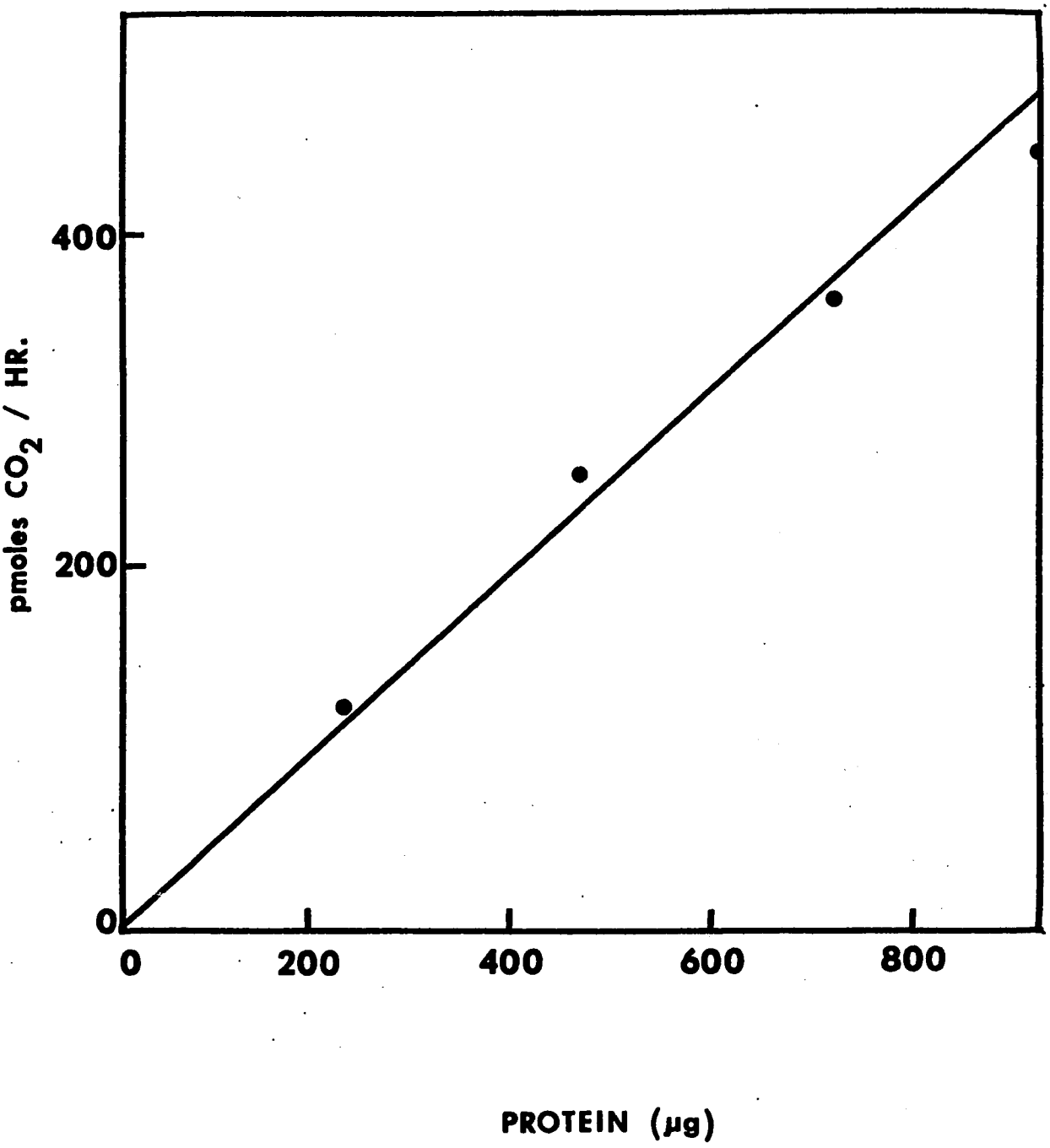
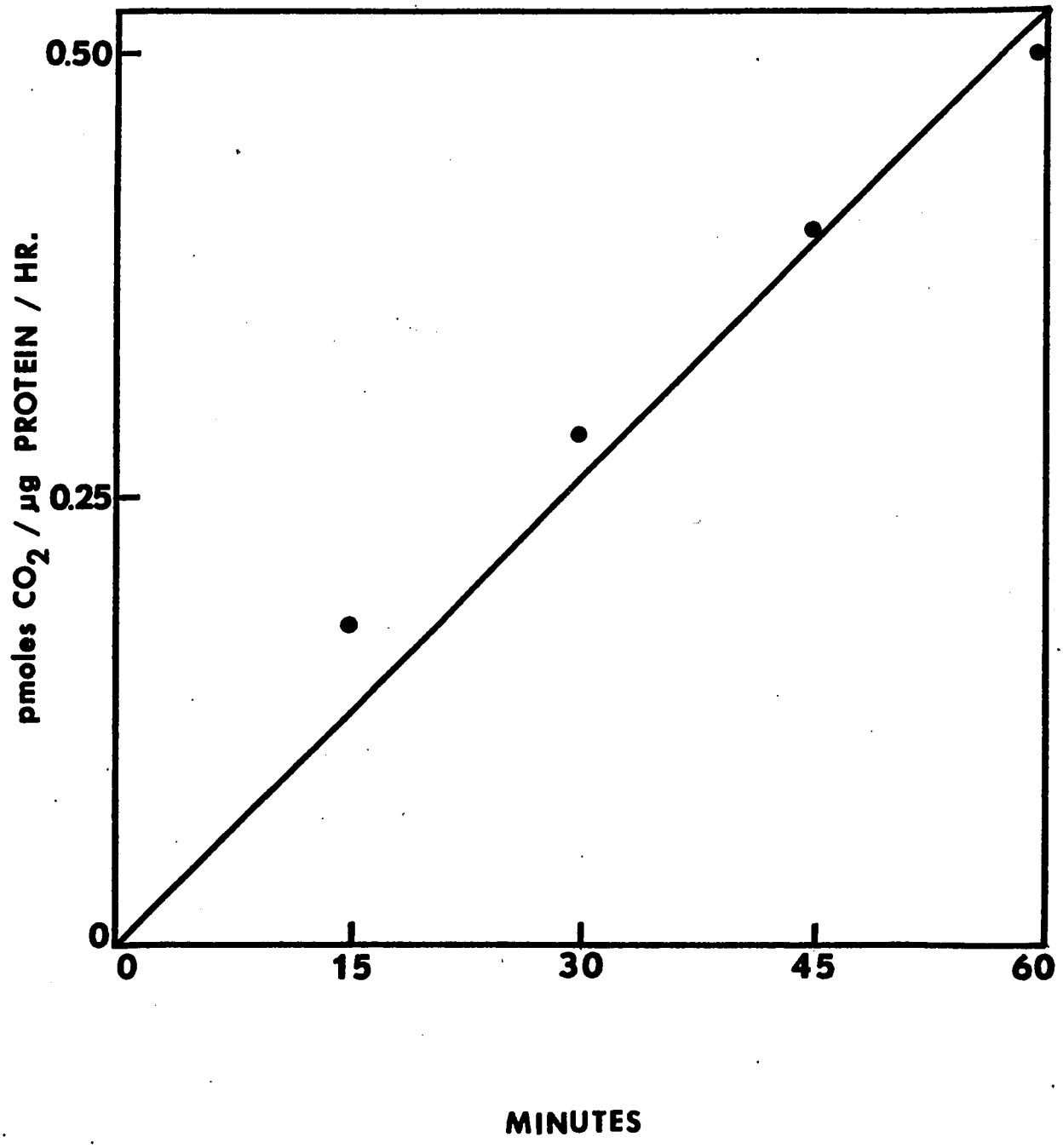


Figure 9. Effect of increasing incubation time on SAMD activity. Each assay contained 0.1 ml of supernatant from an homogenate of 40 ± 3 hour Drosophila larvae (see Materials and Methods). The SAMD assays were performed in a total volume of 0.25 ml consisting of 100 mM sodium-phosphate, pH 7.0; 0.04 mM pyridoxal-phosphate; 0.1 μCi ^{14}C-S-adenosyl-L-methionine; and a total S-adenosyl-L-methionine concentration of 0.2 mM. The decarboxylation reaction was allowed to proceed at 37° for 15, 30, 45, and 60 minutes before the addition of 1 N H_2SO_4.

FIG. 9



representative of enzyme concentrations (Table XI). SAMD activity was assayed with and without added putrescine (2.0 mM).

ODC activity was very high in 24-hour Drosophila larvae. The activity was 14 times greater than the highest reported ODC activity in Cecropia pupae (in wing tissue 16 hours after injection of ecdysone (24)). The level of ODC activity in 24-hour larvae was also greater than the reported activity in the rat prostate (46), liver (11), embryo (11), and in P. polycephalum (56).

ODC activity is still high in 48-hour larvae; however, by 72 hours ODC activity has fallen to 20-25% of its earlier level where it remains in 96-hour Drosophila larvae.

SAMD activity, assayed without exogenous putrescine, was also greatest in 24-48 hour larvae and fell by 30-40% in 72-96 hour Drosophila larvae. The putrescine-stimulated SAMD activity in 48-96 hour larvae was 7-20 times the activity obtained in the absence of putrescine and was greater than SAMD activity similarly assayed in the rat prostate (98), liver (55), brain (57), chick embryo (12), and similar to the SAMD activity observed in the sea urchin (S. purpuratus) (100).

SAMD from 24-hour Drosophila larvae did not exhibit the large putrescine stimulation present in the older larvae studied. SAMD activity was only 30% higher when assayed in the presence of 2.0 mM putrescine. Mitchell and Rusch (56) have also found, in P. polycephalum, that SAMD is not significantly stimulated by putrescine.

TABLE XI

VARIATION IN ODC AND SAMD ACTIVITIES DURING THE DEVELOPMENT OF DROSOPHILA LARVAE.

Drosophila larvae of the indicated ages (± 4 hours) were washed, homogenized, and the ODC and SAMD activities assayed according to Materials and Methods. SAMD activity was determined in the presence and in the absence of 2.0 mM putrescine. (Activity = pmoles CO_2/µg protein/hour.)

Age	ODC activity	SAMD activity	
			+2.0 mM P
24	7.27	0.460	0.596
48	5.21	0.500	10.02
72	1.71	0.182	1.30
96	1.79	0.210	1.60

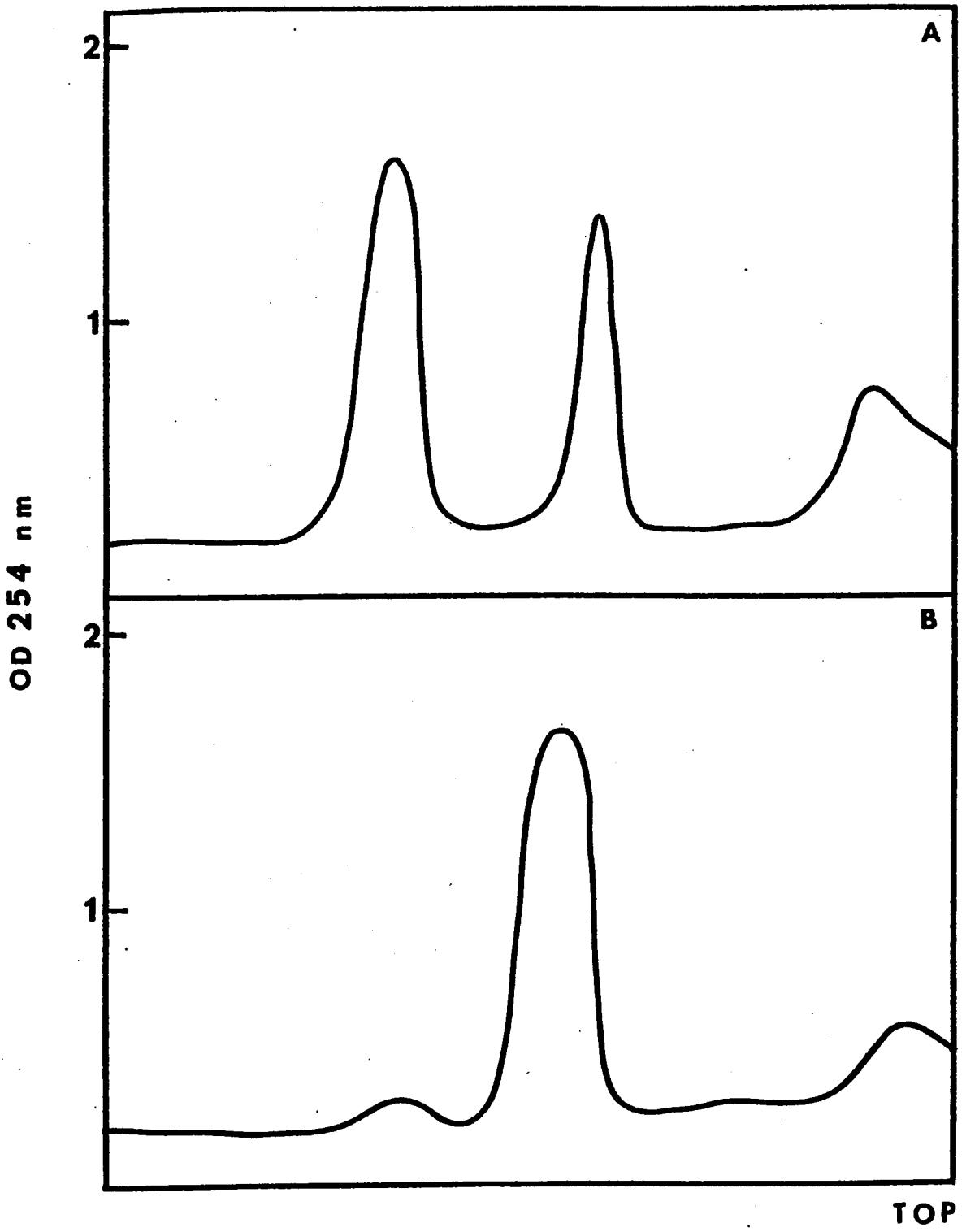
C. Thermolability and Cesium Chloride Extraction of RNA

Although the 26-28S rRNA species from many insects have been shown to be thermolabile (see Literature Review), no one has previously observed the thermolability of D. melanogaster larval RNA. Drosophila larvae were washed and homogenized at 0° in Greenberg-SDS buffer and phenol and the RNA was extracted as described in Materials and Methods. The purified RNA was either put directly onto a sucrose gradient or first heated and then layered onto a gradient (Fig. 10). The unheated larval RNA exhibited the normal 4S, 18S, and 28S peaks with a 28S to 18S ratio of 2:1 and a relatively small 4S peak. The heated RNA had a similar 4S peak; however, the 18S peak was greatly increased while the 28S species was barely detectable. Upon heating, the sedimentation coefficient of the 18S RNA increased to about 20S. Greenberg (65) has also observed a slightly greater sedimentation coefficient of 18S RNA from D. virilis larvae after heating.

Purified 28S rRNA from Drosophila larvae when heated at 60° for 45 seconds was converted entirely to an "18S" product when separated in a sucrose gradient (Fig. 21) or acrylamide gel (Fig. 20). Heated 18S Drosophila larval RNA sedimented slightly faster in sucrose gradients and migrated the same distance as unheated 18S RNA on acrylamide gels. It does not appear that this thermolability is acquired at some stage of larval development since 28S rRNA's extracted from 24-96 hour Drosophila larvae were all found to be converted to an "18S" product upon heating. Shine and Dalgarno (61)

Figure 10. Effect of heat upon Drosophila larval RNA. 72-hour Drosophila larvae were homogenized at 0^{o} in Greenberg buffer-phenol and the RNA extracted as described in Materials and Methods. Following ethanol precipitation, the RNA was dissolved in (A) 0.3 ml of NET-0.5% SDS buffer at room temperature or (B) 0.3 ml of redistilled water, heated at 60^{o} for 45 seconds, and placed into an ice bath. The RNA samples were layered onto 5-30% linear sucrose gradients in NET-0.5% SDS and centrifuged in Beckman's SW 27.1 rotor for twelve hours at 27,000 rpm and 22^{o}. The gradients were fractionated and the absorbance at 254 nm continuously monitored. Centrifugation is from right to left.

FIG. 10



have observed that the 28S RNA from adult flies of D. melanogaster is also thermolabile.

It was possible that the RNA extraction procedure was damaging the 28S rRNA during isolation. For this reason, chick polyribosomes (see Materials and Methods) were homogenized with Greenberg-SDS buffer and phenol and the RNA extracted as previously described (Fig. 11). The absorbance profiles of the heated and unheated RNA were almost identical with only a slight shift in S-value of the heated 28S species. The extraction procedure was capable of isolating thermostable RNA.

An experiment was performed to determine if a break in Drosophila 28S rRNA was caused by the action of larval nuclease during homogenization. Labeled RNA from Drosophila larvae was coextracted with unlabeled RNA from chick polyribosomes. Half of the RNA was heated and gel-electrophoresis of both the heated and unheated RNA performed on 2.7% acrylamide gels (Fig. 12). The unheated RNA (Fig. 12A) has 18S and 28S cpm peaks contributed by the labeled Drosophila RNA and 18S and 28S absorbance profiles contributed by both the chick polyribosome RNA and the Drosophila RNA. However, after heating (Fig. 12B) there was complete conversion of the labeled 28S Drosophila peak to an "18S" product yielding a large 18S cpm peak and a relative increase in the 18S absorbance profile. The unlabeled 28S chick polyribosome RNA remains unaffected by the elevated temperature (see Fig. 11) indicating that the break in the 28S nucleotide chain is not caused by a nuclease released during extraction.

Figure 11. Effect of heat upon chick polyribosome RNA. RNA was extracted at 0^{o} from chick polyribosomes using the Greenberg buffer-phenol procedure outlined in Materials and Methods. Following ethanol precipitation the RNA was dissolved in (A) 0.3 ml of NET-0.5% SDS buffer at room temperature or (B) 0.3 ml redistilled water, heated at 60^{o} for 45 seconds, and placed in an ice bath. The RNA samples were layered onto 5-30% linear sucrose gradients in NET-0.5% SDS and centrifuged in Beckman's SW 27.1 rotor for twelve hours at 27,000 rpm and 22^{o}. The gradients were fractionated and the absorbance at 254 nm continuously monitored. Centrifugation is from right to left.

FIG. 11

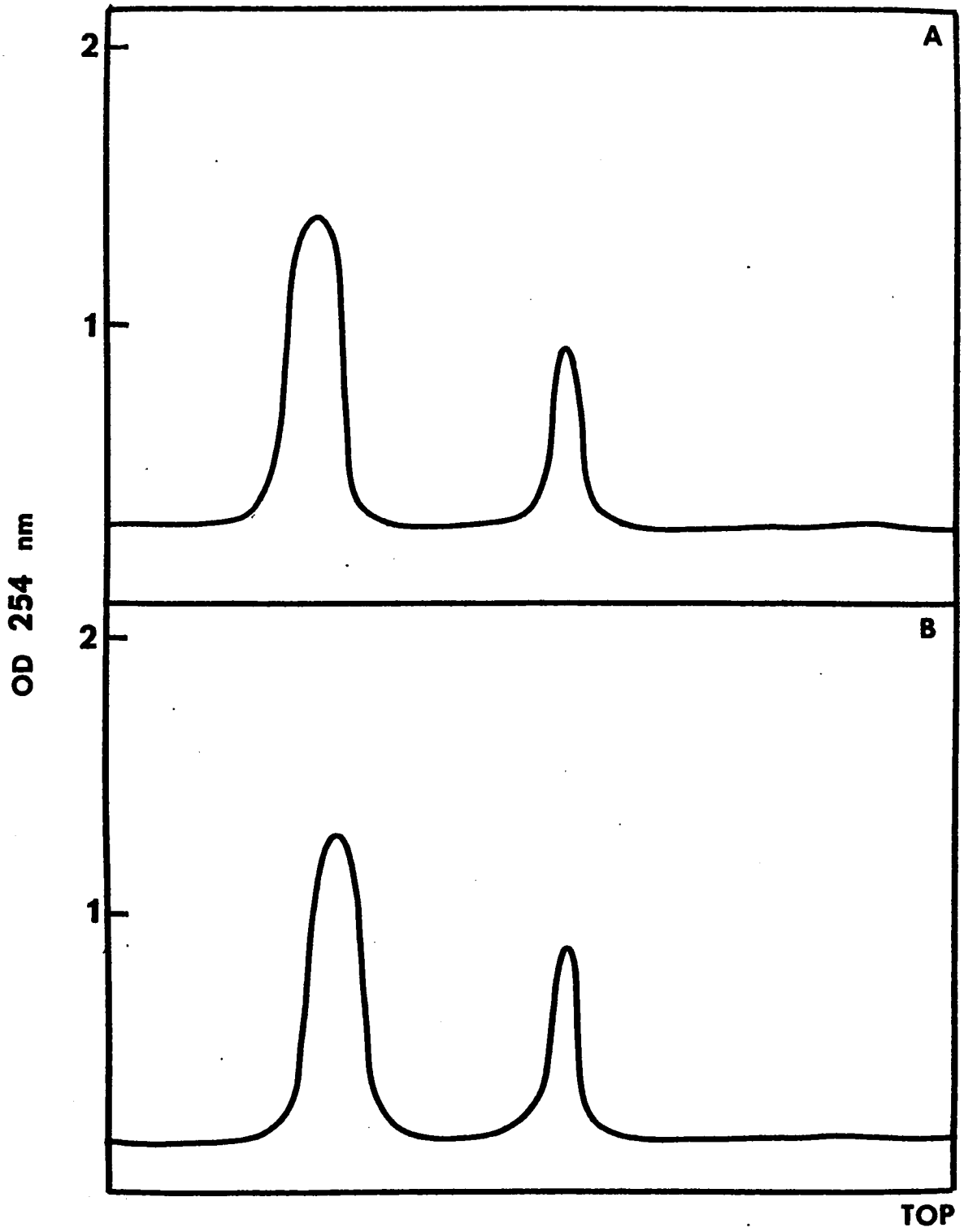
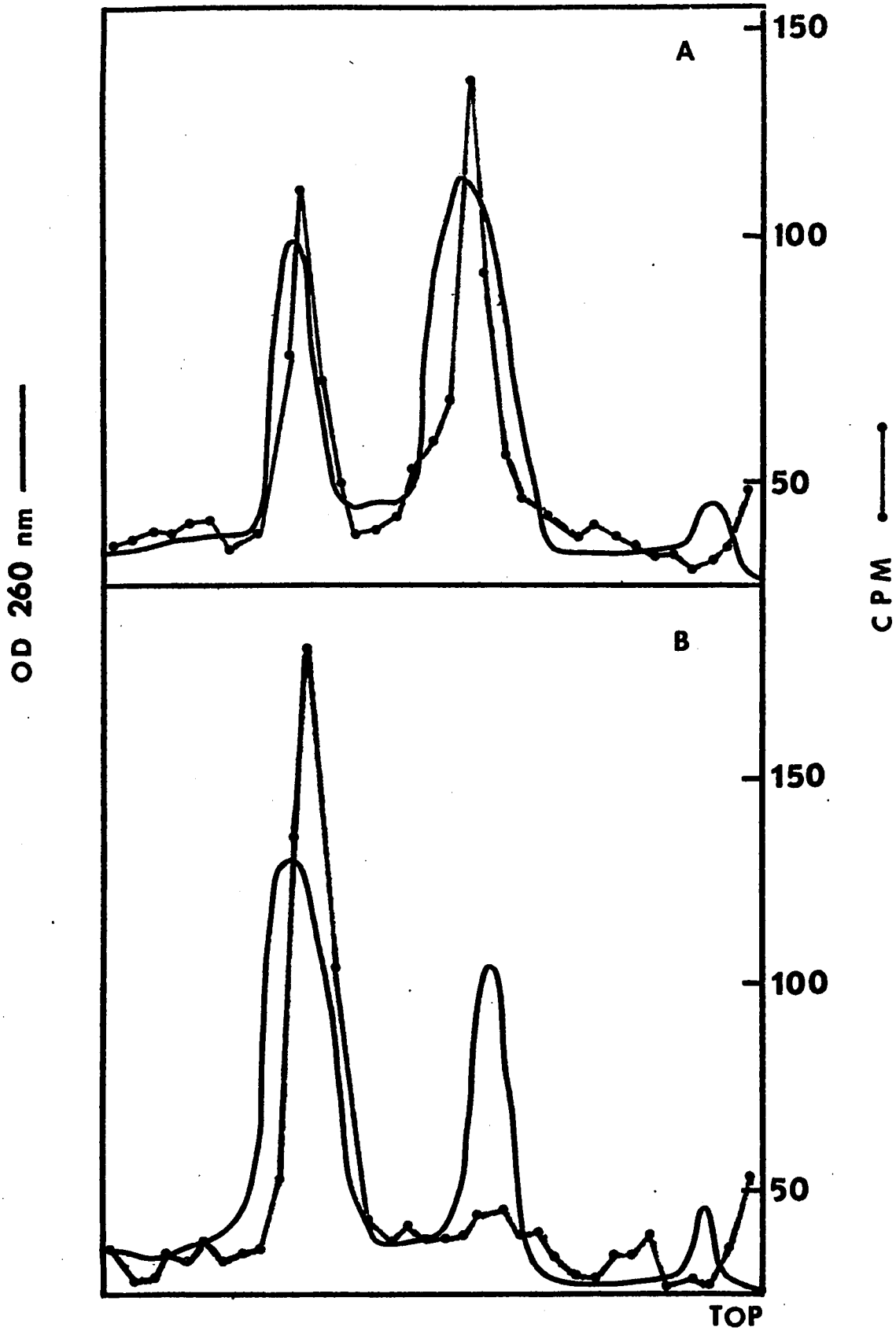


Figure 12. Labeled Drosophila RNA extracted with unlabeled chick polyribosome RNA. 72-hour Drosophila larvae were incubated in liquid culture media with ^{3}H-uridine, washed, and homogenized at 0° with unlabeled chick polyribosomes in Greenberg buffer-phenol. The RNA was extracted as described in Materials and Methods and, following ethanol precipitation, was dissolved in (A) SDS-gel buffer with 10% (w/v) sucrose or (B) redistilled water and heated at 60° for 45 seconds, cooled rapidly, and an equal volume of (2X) SDS-gel buffer with 20% (w/v) sucrose added. 1.5 O.D. 260 of each sample was layered onto 2.7% acrylamide gels and electrophoresed at 5 ma/tube for 2.5 hours. The gels were scanned at 260 nm in a Joyce-Loebl Chromoscan and sliced into 1.5 mm sections. The slices were dissolved in 30% H_2O_2 and counted in Aquasol. Electrophoresis is from right to left. The small O.D. peak at the top of the gel is DNA. (For details of gel-electrophoresis procedure, see Materials and Methods.)

FIG. 12



RNA has been extracted from various tissues using a new CsCl procedure (for details, see Materials and Methods). Figure 13 shows the absorbance spectrum of CsCl-extracted RNA from two sources. The RNA from sea urchin eggs and from Drosophila larvae appears to be relatively protein-free as evidenced by the 260 to 280 ratio of 2.0 and 2.2 respectively.

CsCl extraction yielded 10-20% more total RNA (O.D. 260) than did phenol-extraction of the same wet weight of sea urchin eggs or Drosophila larvae. Recovery of purified 16S and 23S E. coli RNA re-extracted with the CsCl procedure was 95-100%. However, the addition of one mg of bovine serum albumin per 100 µg of E. coli RNA prior to extraction resulted in a 20% loss of the added RNA. In all cases the RNA following CsCl extraction was free of DNA.

The absorbance profiles following sucrose-gradient centrifugation of CsCl-extracted RNA from three different sources are shown in Figures 14 and 15. Drosophila larval RNA isolated in this manner has much more 28S rRNA relative to the 18S species (Fig. 14A) than does phenol-extracted RNA (Fig. 11A). The relative amount of 28S RNA from CsCl-extracted sea urchin eggs also greatly increased (Fig. 14B). Phenol-extracted RNA from chick polyribosomes has a 28S to 18S ratio of 2:1 (Fig. 15A). However, this ratio is increased to 3:1 in CsCl - extracted polyribosomes (Fig. 15B). Similar proportions of RNA were also observed in acrylamide-gel separations.

Figure 13. Spectrum of RNA from Drosophila larvae and sea urchin eggs isolated by the CsCl procedure. The absorbance of RNA from 72-hour Drosophila larvae (- - - - - -) and sea urchin eggs (———) isolated using the CsCl procedure outlined in Materials and Methods was determined from 220-290 nm. The RNA was dissolved in NET buffer and the absorbance recorded against an appropriate blank every 5 nm in a Beckman DU-2 spectrophotometer. The absorbance is shown as a continuous tracing.

FIG. 13

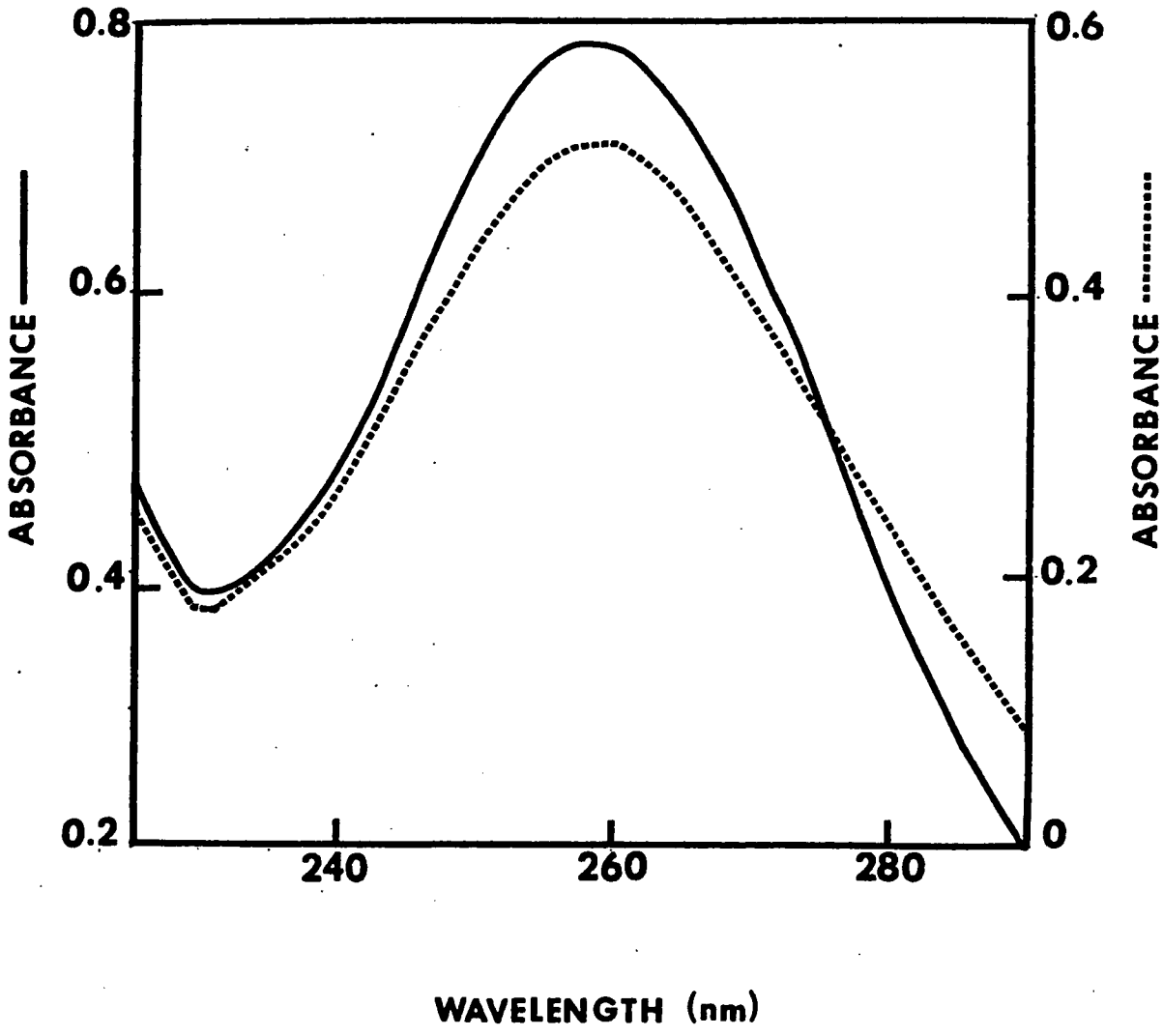

0.8
0.6
0.4
0.2
ABSORBANCE
0.6
0.4
0.2
0
ABSORBANCE
240
260
280
WAVELENGTH (nm)

Figure 14. CsCl extraction of RNA from Drosophila larvae and sea urchin eggs. The RNA from 72-hour Drosophila larvae (A) and sea urchin eggs (B) was extracted using the CsCl procedure outlined in Materials and Methods. The RNA was reprecipitated from ethanol-salts, dissolved in 0.3 ml NET-0.5% SDS buffer and layered onto 5-30% sucrose gradients in NET-0.5% SDS. Centrifugation was in Beckman's SW 27.1 rotor at 27,000 rpm for twelve hours at 22°. The gradients were fractionated and the absorbance at 254 nm continuously monitored. Centrifugation is from right to left.

FIG. 14

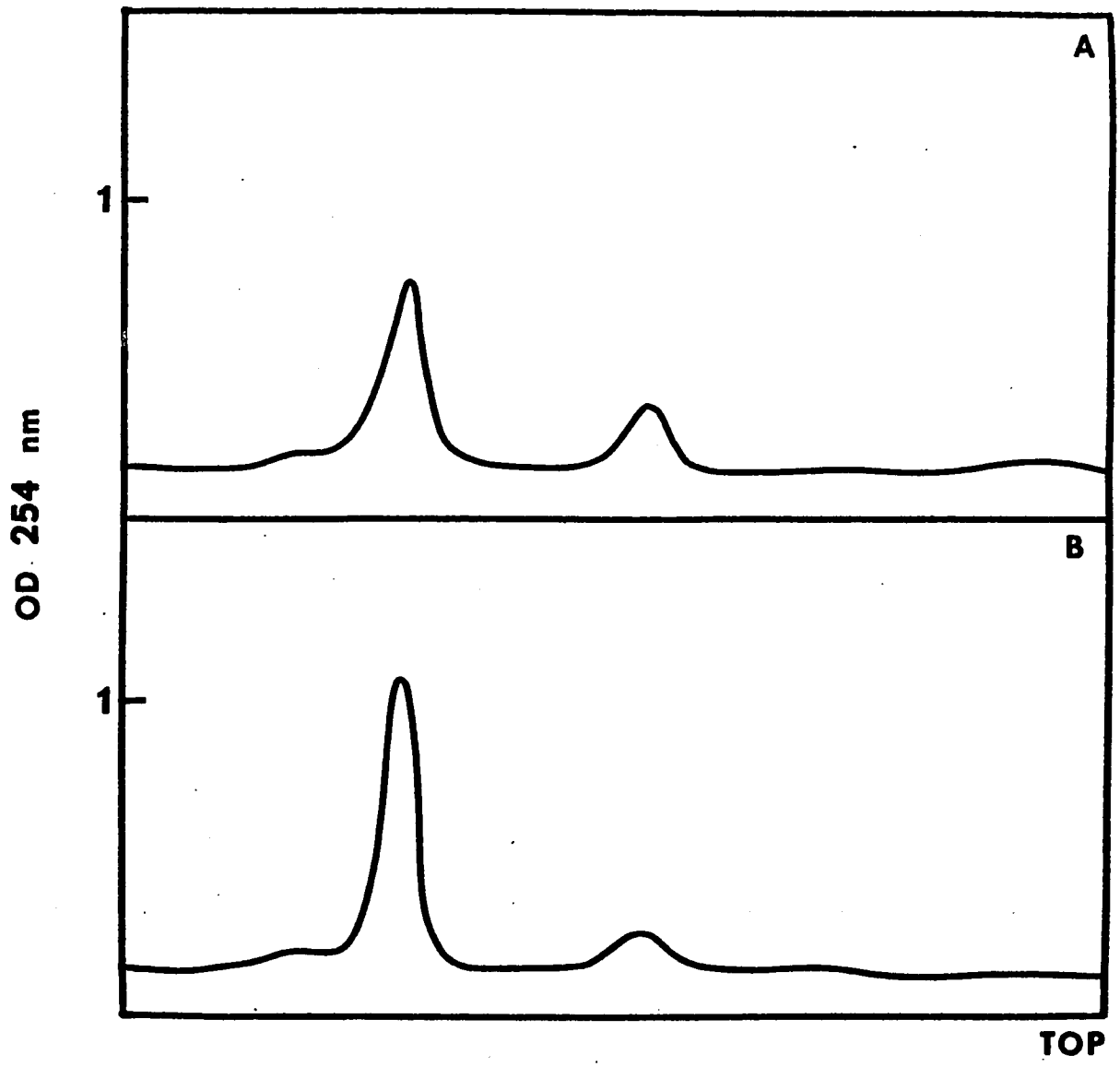
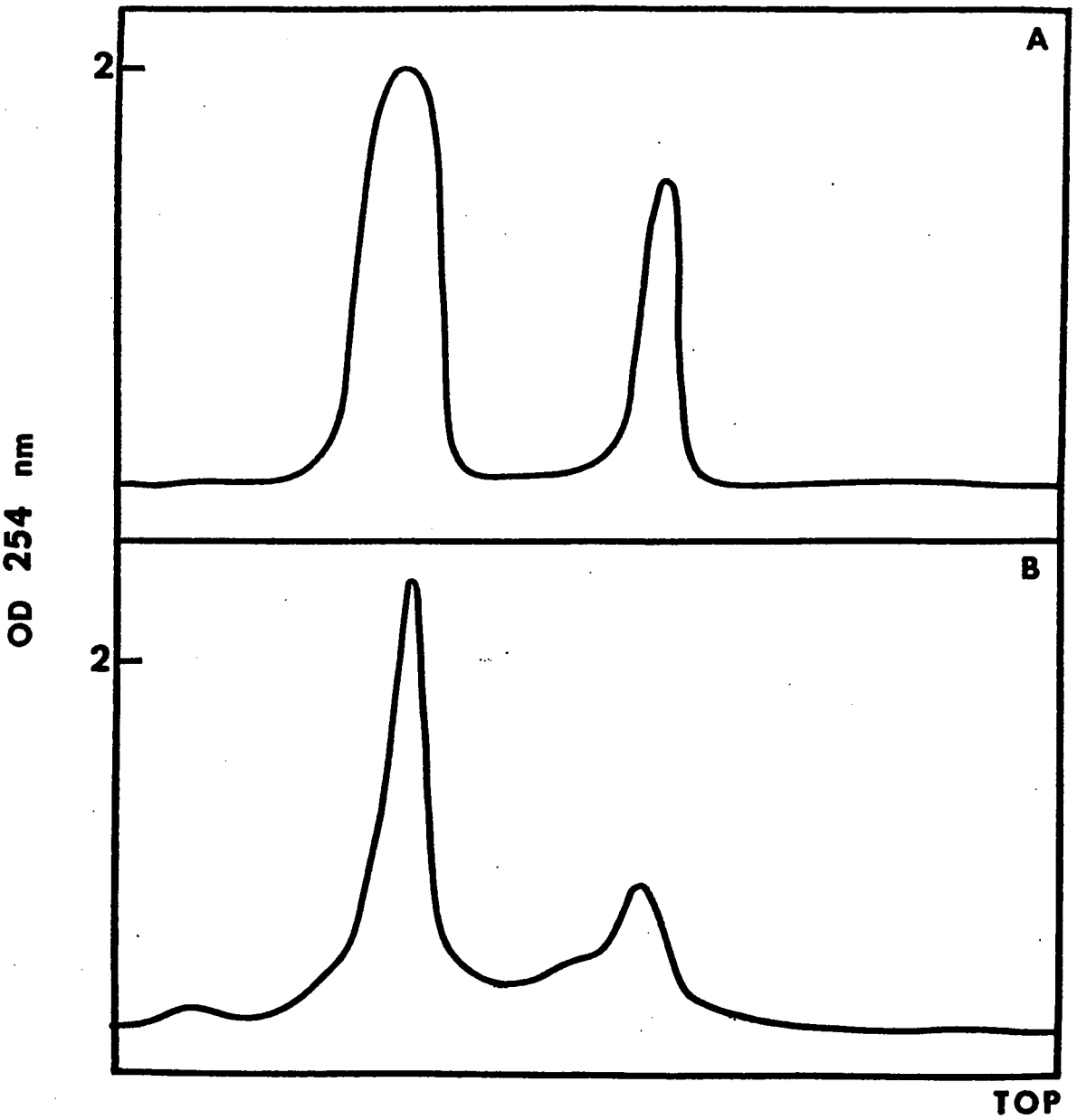


Figure 15. Comparison of RNA extracted from chick polyribosomes by phenol and CsCl procedures. RNA was extracted from chick polyribosomes using either (A) the Greenberg buffer-phenol procedure or (B) the CsCl procedure outlined in Materials and Methods. Following precipitation in ethanol-salts, the RNA was layered onto 5-30% sucrose gradients in NET-0.5% SDS and centrifuged in Beckman's SW 27.1 rotor at 27,000 rpm for twelve hours at 22°. The gradients were scanned at 254 nm. Centrifugation is from right to left.

FIG. 15



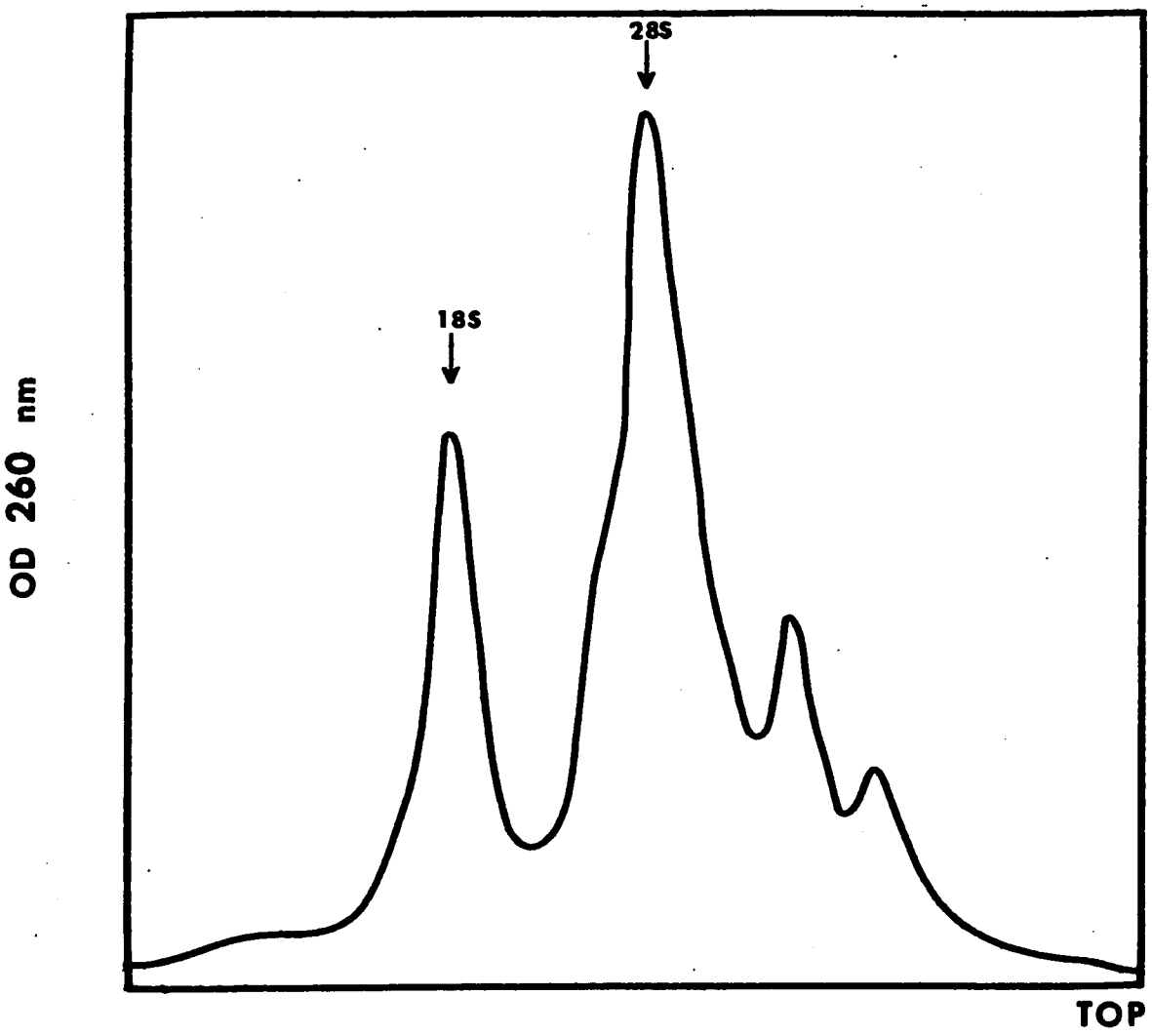
The relative increase in the amount of 28S RNA was assumed to be due to aggregation of the 18S rRNA species during CsCl extraction. There were several factors which could have been causing aggregation. The addition of 100 mM EDTA to the CsCl-Sarkosyl buffer and/or to the CsCl cushion to eliminate magnesium ions did not prevent aggregation. The high ionic strength of the CsCl gradient was thought to contribute to an increased degree of RNA-RNA interaction. However, phenol-extracted Drosophila larval RNA incubated for twelve hours in either the CsCl-Sarkosyl buffer or the higher concentration of CsCl found in the cushion (density of 1.707) did not result in any appreciable aggregation of the RNA species.

Phenol-extracted Drosophila larval RNA passed through a CsCl gradient did aggregate substantially (Fig. 16). The 18S and 28S peaks were present, but the 28S to 18S ratio was increased to 3:1, similar to larval RNA extracted initially with CsCl (Fig. 14A). Two peaks migrating more slowly than 28S were also present.

Phenol-extracted Drosophila larval RNA pelleted through 10% sucrose in NET-0.5% SDS under similar conditions to the CsCl centrifugation (40,000 rpm at 25^{o} for twelve hours) did not aggregate. From the above observations, the aggregation of the RNA during the CsCl isolation procedure was believed to be caused primarily by the high concentration of RNA in the pellet due to the force exerted during centrifugation and, in part, by the high ionic strength of the

Figure 16. Phenol-extracted Drosophila larval RNA passed through a CsCl gradient. The RNA from 72-hour Drosophila larvae was extracted at 0^{o} with Greenberg-SDS buffer and phenol as described in Materials and Methods. The RNA was then mixed with 4 ml of 4% Sarkosyl buffer and 4 gm of solid CsCl added. Four ml of this solution was layered onto 1.2 ml of a CsCl-cushion in a centrifuge tube and centrifuged in Beckman's SW 50L rotor for twelve hours at 35,000 rpm at 25^{o}. The pelleted RNA was reprecipitated from ethanol-salt and 1.5 O.D. 260 units electrophoresed on 2.7% acrylamide gels at 5 ma/tube for 2.5 hours. The gels were scanned at 260 nm with phenol-extracted larval RNA run on duplicate gels as markers. (For details of the CsCl and gel-electrophoresis procedures, see Materials and Methods.) Electrophoresis is from right to left.

FIG. 16



CsCl solution. These conditions existing during CsCl extraction provided an interesting opportunity to study several aspects of RNA-RNA interactions.

All of the thermolability studies with insect RNA have been performed with RNA extracted with phenol (60-65). In order to determine if phenol was specifically causing insect 28S rRNA to become thermolabile, Drosophila larval RNA was extracted via CsCl and heated to 60^{o} for 45 seconds (Fig. 17). The large 28S peak present in CsCl-extracted RNA nearly disappeared, leaving a large 18S peak comparable to heated phenol-extracted larval RNA (Fig. 10 and 12).

In order to determine if 18S rRNA would aggregate yielding larger RNA species, purified 18S rRNA from Drosophila and chick polyribosomes were centrifuged through CsCl (Fig. 18 and 19). The Drosophila 18S RNA did aggregate to form an RNA species with a larger sedimentation coefficient (27S) in both a sucrose-gradient and gel (Fig. 18). Although the "aggregate" was present in a small amount, it was detectable and not a contaminant of 28S rRNA. However, 18S rRNA from chick polyribosomes aggregated to a much greater extent (Fig. 19A) (believed to be due to the higher concentration of RNA passed through CsCl). The largest aggregate of 18S RNA sedimented slightly slower than native 28S rRNA. There were also two smaller peaks migrating faster than the 28S marker. This is comparable to the two larger RNA species seen in the gel-electrophoresis of purified Drosophila RNA after CsCl centrifugation shown in Fig. 16. The remaining unaggregated chick

Figure 17. Thermolability of CsCl-extracted Drosophila larval RNA. 72-hour Drosophila larvae were homogenized in 10-30 volumes of 4% Sarkosyl buffer and the RNA isolated via the CsCl procedure outlined in Materials and Methods. The RNA was precipitated from ethanol-salts, dissolved in redistilled water, heated to 60° for 45 seconds, and rapidly cooled to 0°. The RNA was layered onto 5-30% sucrose gradients in NET-0.5% SDS and centrifuged in Beckman's SW 27.1 rotor at 27,000 rpm for twelve hours at 22°. The gradient was fractionated and the absorbance at 254 nm continuously monitored. Centrifugation is from right to left. (Phenol-extracted Drosophila larval RNA was centrifuged on an identical gradient as a marker.)

FIG. 17

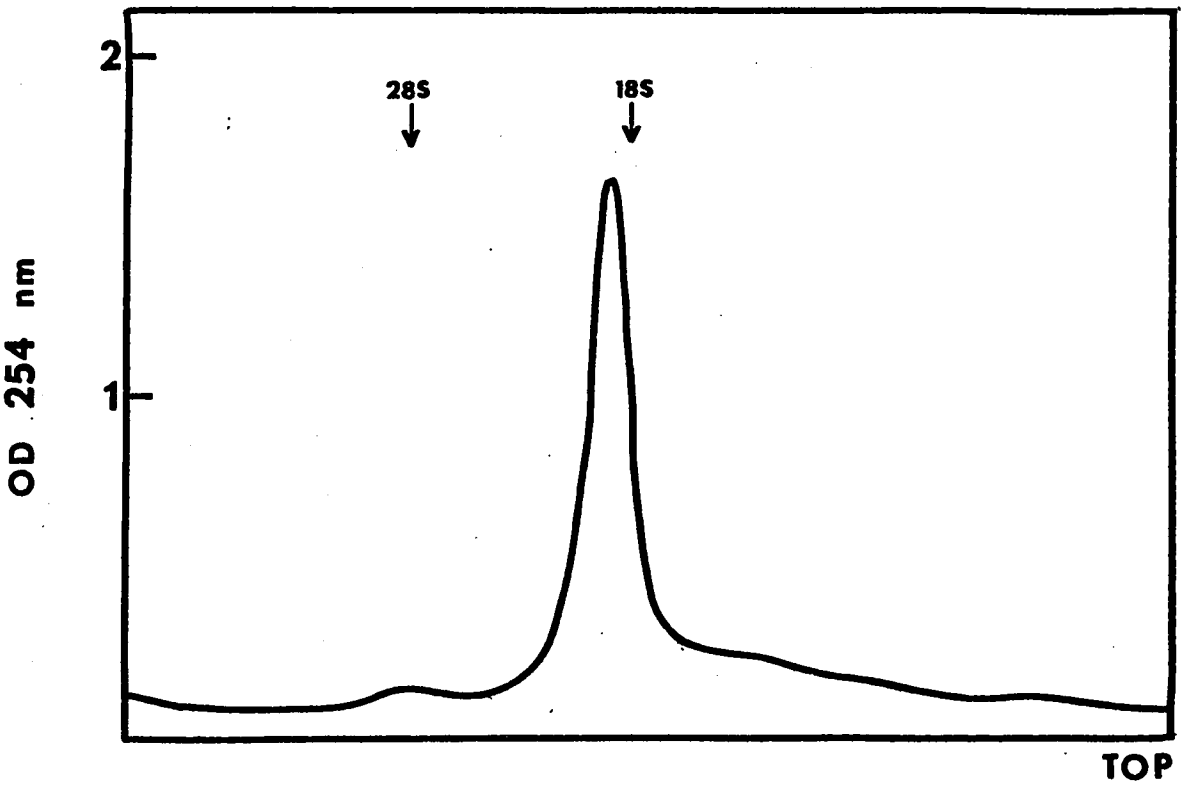


Figure 18. Purified 18S Drosophila larval RNA passed through CsCl. 72-hour Drosophila larvae were homogenized at 0° with Greenberg-SDS buffer and phenol and the RNA extracted as indicated in Materials and Methods. The RNA was fractionated on a sucrose gradient, the 18S species isolated and precipitated in ethanol-salts. The purified 18S RNA was mixed with 4 ml of 4% Sarkosyl buffer, 4 gm CsCl was added, and the solution layered onto a 1.2 ml CsCl cushion. Following centrifugation in Beckman's SW 27.1 rotor at 35,000 rpm for twelve hours at 25°, the pelleted RNA was precipitated from ethanol-salts and (A) electrophoresed in 2.7% acrylamide gels at 5 ma/tube for 2.5 hours, or (B) layered onto a 5-30% sucrose gradient in NET-0.5% SDS and centrifuged in the SW 27.1 rotor at 27,000 rpm for twelve hours at 22°. The gradient was fractionated and the absorbance at 254 nm continuously monitored. The gel was scanned at 260 nm. Phenol-extracted larval RNA was run in a duplicate gradient and gel as a marker. Gel and gradient separations of the purified 18S RNA prior to CsCl centrifugation revealed no detectable contamination by the 28S species. Centrifugation and electrophoresis are from right to left. (A and B represent two separate experiments.)

FIG. 18

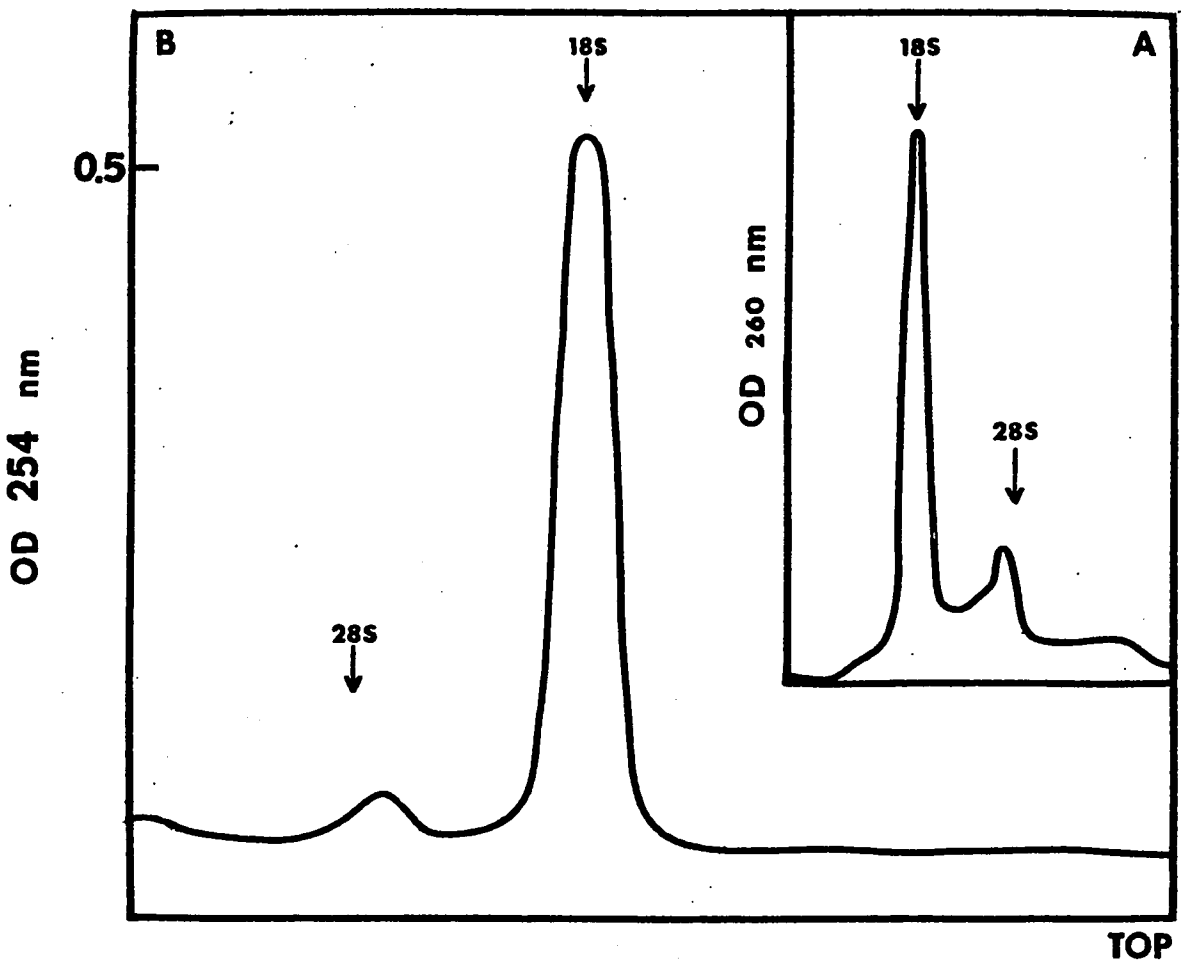
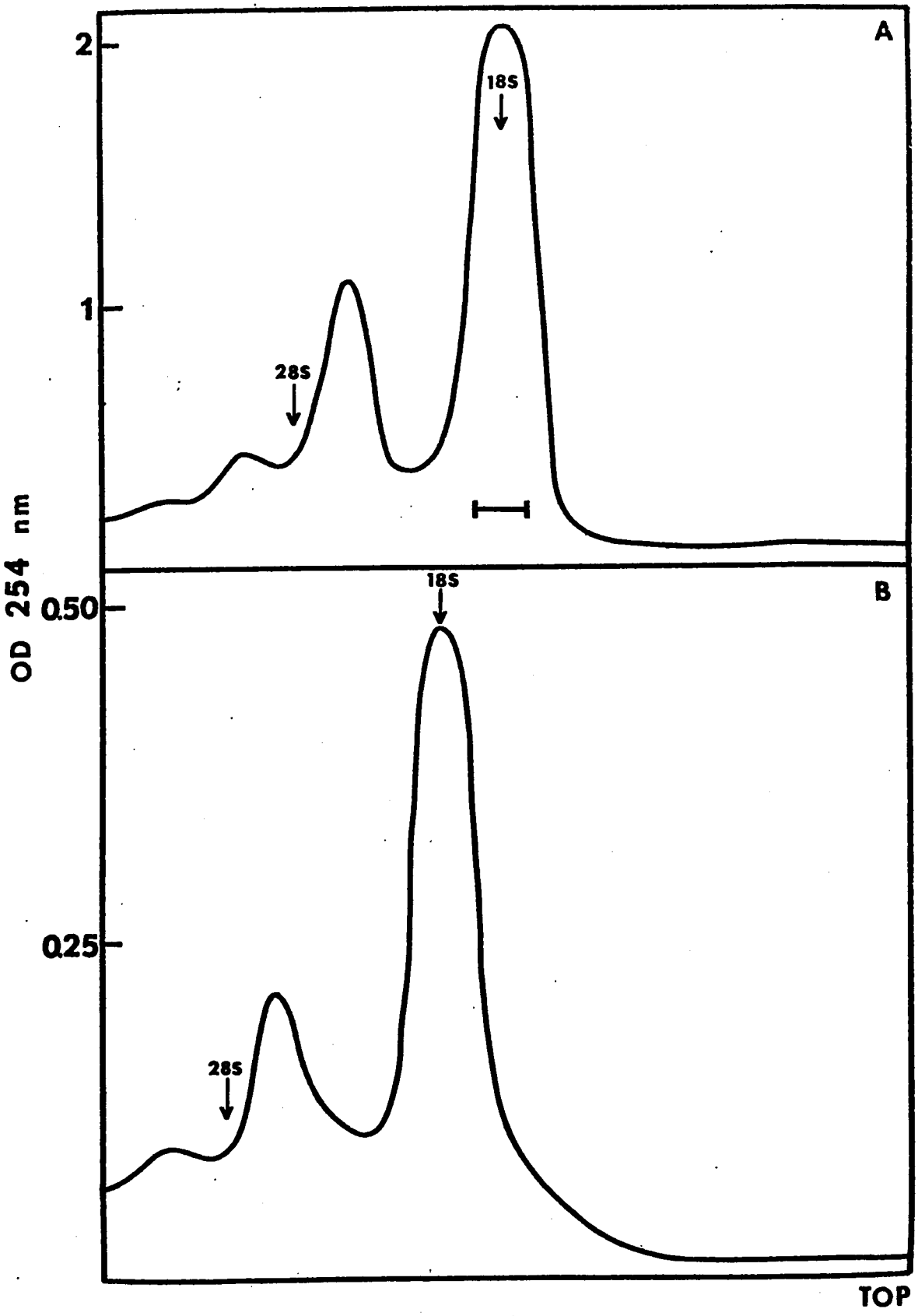


Figure 19. Purified 18S chick polyribosome RNA passed through CsCl. 18S chick polyribosome RNA was extracted and purified as explained in the legend to Fig. 18. Contamination of 28S RNA was less than 1%. The 18S RNA was passed through CsCl and the pelleted RNA layered onto 5-30% sucrose-gradients in NET-0.5% SDS. Centrifugation was at 27,000 rpm for twelve hours at 22°. The gradient (A) was fractionated and the absorbance at 254 nm continuously monitored. The fractions under the center of the 18S peak were collected and the RNA precipitated in ethanol-salts. The 18S RNA was again passed through CsCl and fractionated on a sucrose-gradient (B) under similar conditions. (Centrifugation was for thirteen hours.) Phenol-extracted chick polyribosome RNA run in identical gradients was used as a marker. Centrifugation is from right to left.

FIG. 19



polyribosome 18S RNA centrifuged for a second time through CsCl again aggregated to form larger RNA species (Fig. 19B). (Phenol-extracted 28S Drosophila RNA did not aggregate in CsCl.)

Several authors (61,63) have indicated their inability to reassociate the "18S" product from heated 28S insect rRNA. Figures 20 and 21 show the results of two experiments attempting to reanneal heated Drosophila 28S rRNA by centrifugation through CsCl. In both cases, significant amounts of a 28S RNA were obtained after centrifugation of heated 28S rRNA through CsCl. The reaggregated RNA traveled the same distance as marker 28S rRNA in both gels and gradients. The gel separation (Fig. 20) also revealed the presence of a small amount of RNA not migrating as far as the "28S" aggregate.

Figure 20. Reannealing of heated 28S Drosophila larval RNA. 28S RNA was purified from phenol-extracted 72-hour Drosophila larval RNA as indicated in Materials and Methods and in the legend to Fig. 18. The 28S rRNA was dissolved in water and heated at 60° for 45 seconds, cooled to 0°, and 1 O.D. 260 electrophoresed on 2.2% acrylamide gels at 5 ma/tube for three hours. The gel was scanned at 260 nm (A). The remainder of the heated 28S RNA was passed through CsCl, precipitated from ethanol-salts, and 1 O.D. 260 electrophoresed and scanned in the same manner (B). Electrophoresis is from right to left.

FIG. 20

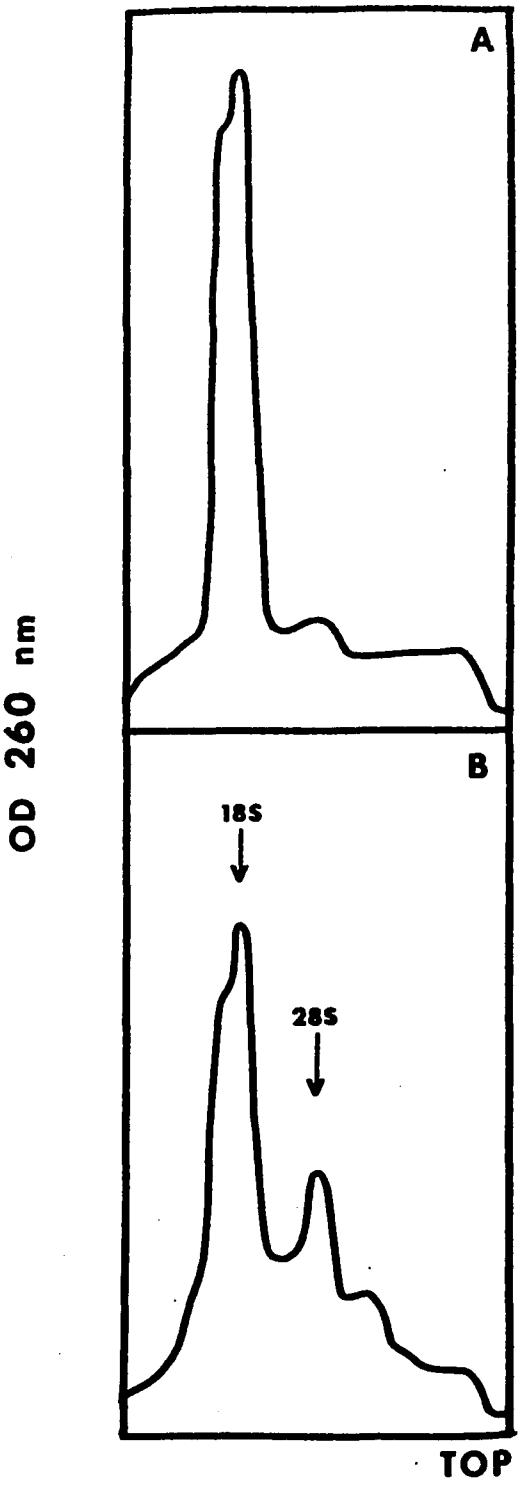
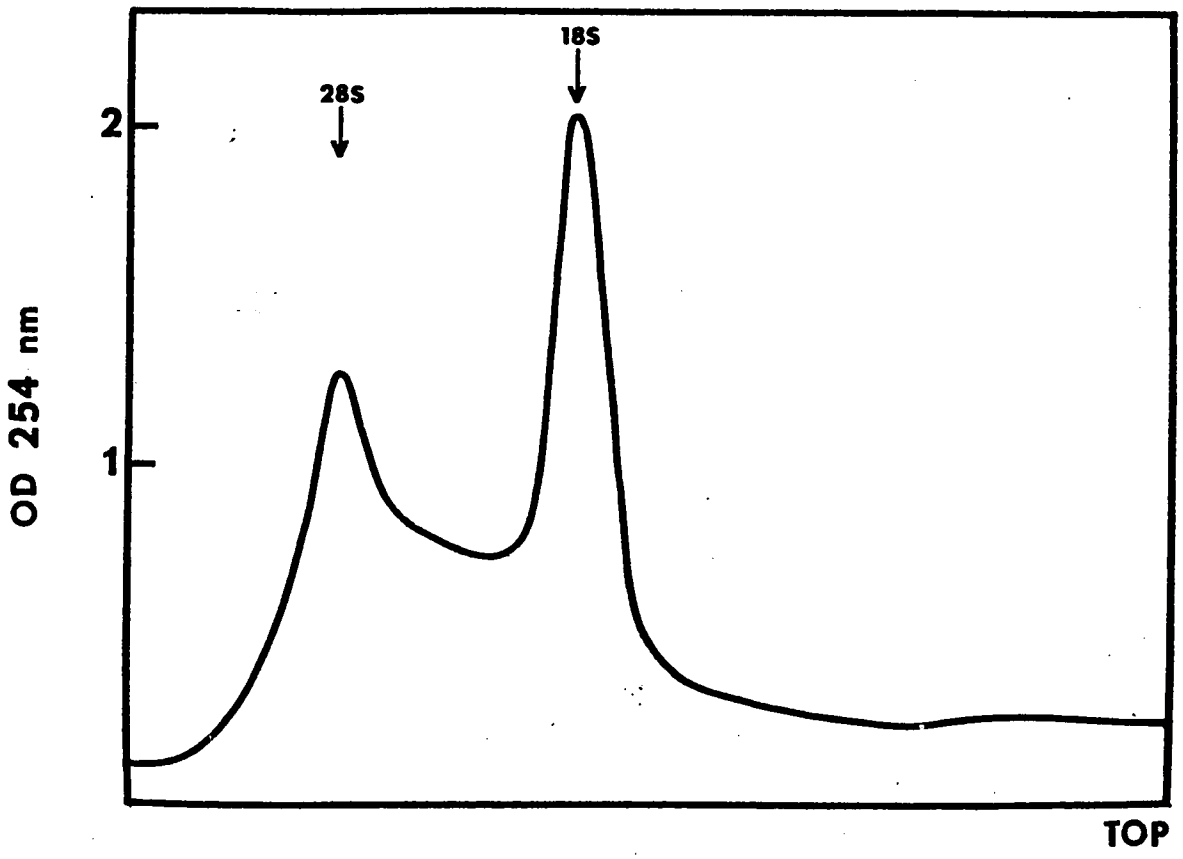


Figure 21. Reannealing of heated 28S Drosophila larval RNA. The experiment performed was similar to that outlined in the legend to Fig. 20. However, after passage through CsCl, the heated 28S rRNA was separated on a 5-30% sucrose-gradient in NET-0.5% SDS. Centrifugation was in Beckman's SW 27.1 rotor for twelve hours at 27,000 rpm and 22°. (Cleavage of the 28S RNA following heating was complete as evidence by gel-electrophoresis.) Centrifugation is from right to left. Phenol-extracted larval RNA was run in a duplicate gradient as a marker.

FIG. 21



DISCUSSION

A. Stimulation of RNA Synthesis by Spermidine

Spermidine was shown to cause increased incorporation of ^{3}H-uridine into the RNA of 72-hour *Drosophila* larvae. This was a general stimulation with the specific activities of 4S, 18S, and 28S RNA all being increased. The degree of stimulation was observed to be related to the time of incubation and the concentration of spermidine. The specificity of spermidine, among the related amines, in its ability to increase the incorporation of ^{3}H-uridine was also shown. The incorporation of ^{3}H-thymidine into DNA was not affected, while spermidine increased slightly the incorporation of ^{14}C-L-leucine into total protein of *Drosophila* larvae after eight hours of incubation.

All of the amines investigated, except cadaverine, inhibited incorporation of ^{3}H-uridine at high concentrations while putrescine was inhibitory even at low levels (Tables II and III). Raina *et al.* (101) found that 1-5 mM spermidine increased by 50% the specific activity of RNA in Ehrlich ascites cells. However, at concentrations of 10 mM and greater, spermidine inhibited incorporation of ^{3}H-uridine into RNA. Dion (35) has also observed the inhibitory effects of increased levels of spermidine upon the uptake of ^{3}H-uridine by salivary glands of *Drosophila* larvae. The inhibitory effect of high concentrations of amines could be due to the production of oxidized amines by amine oxidases in

Drosophila. Oxidized amines have been observed to inhibit RNA and protein synthesis in both eucaryotic and procaryotic cells (6,102,103). The sera of several animals have been found to contain sufficient amine oxidase activity to produce toxic levels of oxidized amines (6). It is possible that Drosophila hemolymph also has sufficient amine oxidase activity to cause this in the presence of high exogenous amine levels.

There are many possible mechanisms by which the exogenously administered spermidine could be increasing the incorporation of ^{3}H-uridine into larval RNA. Spermidine could cause a greater uptake of labeled uridine and/or increase its conversion to labeled triphosphates used for the synthesis of RNA. The possibility of an elevated specific activity of the radioactive precursor pool has not been previously investigated (10,26-35). Our results (Table V) show that the degree of labeling of the UTP pool in Drosophila larvae was not appreciably affected by incubation with any of the amines studied.

The specific activity of ^{3}H-CTP (also shown to be incorporated into larval RNA--Table IV) was not determined due to the inability of the high-voltage electrophoresis procedure to separate CTP from other nucleotide di- or triphosphates present. However, if the radioactive CTP pool was increased in the presence of spermidine, the relative proportion of labeled cytosine in RNA from larvae incubated with the amine would be greater than in RNA from larvae

incubated without amine. The RNA from both sources had similar proportions of labeled cytosine and uridine (Table IV).

The action of amines to decrease the enzymatic degradation of RNA has also been postulated to be the cause of this increased accumulation of RNA (30,42-44). For this reason, the effect of spermidine on the turnover of the various mature species of larval RNA was determined (Table VI). A decreased rate of turnover of cytoplasmic RNA due to a spermidine inhibition of nuclease action would result in an elevated RNA specific activity. The turnover of 4S, 18S, and 28S RNA was not increased by spermidine (Table VI) during periods of time when spermidine was observed to stimulate the incorporation of ^{3}H-uridine.

The RNA did not appear to turn over in the three and six hours of incubation subsequent to labeling with ^{14}C-L-methionine. Although the half-lives of the various species of Drosophila larval RNA were not found in the literature, the values obtained in other systems (35-45 hours for 4S, 18S, and 28S RNA in cultured fibroblasts (94) and 3.5 days for rRNA in rat liver (95)) indicate that the differences in specific activities of the RNA following a six hour "chase" would be extremely small. Since the RNA content of Drosophila larvae increases rapidly up to pupation (78) and the function of the larval stage of development of endopterogote insects is to store materials (including RNA (96)) needed for further development (9), it is possible that larval RNA does not turn over. Weber (94) has found that rapidly growing cells in culture also possess RNA that does not turn over.

In order to further investigate the possible mechanisms of action of exogenously administered spermidine, attempts were made to determine if the polyamine was stimulating the synthesis of the 38S rRNA precursor found in Drosophila larvae (65). Labeled precursor was not detectable on sucrose gradients or acrylamide gels even when larvae were incubated for fifteen minutes with up to 150 μCi of ^{3}H-uridine. This was probably due to the rapid rate of conversion of Drosophila rRNA precursor (65) and the inability of the larvae to take up enough ^{3}H-uridine in a short time to preferentially label the precursor relative to the other species of RNA. Weinmann (104) was also unable to obtain a distinct 38S rRNA precursor in adult Drosophila following injection of ^{3}H-uridine for short periods. Wylie (33) and Fausto (32) have shown that exogenously administered amine can stimulate the incorporation of ^{3}H-uridine into rRNA precursor in amphibian oocytes and regenerating rat liver.

Exogenously-administered amine in Drosophila larvae and in the other systems studied (see Literature Review) could be causing greater accumulation of RNA by increasing the actual rate of synthesis of RNA or by decreasing the rate of degradation of the precursors to tRNA and rRNA (105). Observations supporting both possibilities have been reported. Added amine has been shown to stimulate DNA-dependent RNA polymerase in a variety of procaryotic and eucaryotic in vitro systems (36-41). Raina and Jänne (29,30) observed that amines decreased the rate of degradation of pulse-labeled RNA

following inhibition of RNA synthesis by actinomycin D. However, the possibility that the amines were affecting the ability of the inhibitor to interfere with the synthesis of RNA (45) was not determined. Although the rate of processing of rRNA precursor has been decreased in cells starved for methionine (106) and incubated with inhibitors of protein synthesis (107), no specific metabolites that increase the rate of processing have been identified.

In order to adequately distinguish between these two possible modes of action of added amine, the actual rate of synthesis of the RNA precursors and their rate of processing to mature RNA would have to be studied. This would involve the estimation of the amounts of precursor present and the specific activity of the radioactive triphosphate pools at several incubation times. Such a study would probably have to be done in a cell culture system similar to the one developed by Emerson (74,92).

Assuming that added amine and endogenous amine have a similar effect on the synthesis of RNA during development, several inferences can be drawn concerning the functions of polyamines. Although there is some evidence indicating that the regulation of processing and degradation of pre-rRNA is a possible control mechanism for rRNA synthesis (108), several investigators have shown in cultured animal cells that the great increases in rates of rRNA production following the reversal of contact-inhibition were due entirely to an increased rate of synthesis of rRNA precursor (74,94). The

control of rRNA synthesis during development in insects also seems to be due to differential rates of precursor synthesis rather than to altered rates of degradation (89,109).

Little is known concerning the control of tRNA synthesis (105). A pre-tRNA molecule larger than mature 4S tRNA has been observed in HeLa cells (105) and in salivary gland cells of C. tentans (110), and the nuclear turnover of the 4S RNA and its precursor is relatively rapid (110). There has also been a postulated mechanism for the control of tRNA synthesis by differential rates of degradation of this precursor in some systems (111).

There is evidence linking elevated levels of polyamines with periods of rapid growth and RNA synthesis (see Literature Review), and exogenously administered amines have been shown to increase the net accumulation of RNA. It is not inconsistent with these observations that polyamines could be functioning in the cell by allowing DNA-dependent RNA polymerase to make more RNA.

B. Ornithine Decarboxylase and S-Adenosyl-L-Methionine Decarboxylase in Drosophila Larvae

L-ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase were found in Drosophila larvae. They exhibited high activities and had properties similar to the corresponding enzymes in other tissues. ODC in Drosophila was activated by DTT (24,46), had increased activity in the presence of pyridoxal-phosphate (46,48), and had an apparent K_m for L-ornithine close to reported values (24). SAMD activity was

slightly inhibited by Tris-HCl (98), had an apparent K_m for S-adenosyl-L-methionine similar to the enzyme in other tissues (55,57,98), and was significantly stimulated by putrescine during most stages of development (51-55,57).

Larvae incubated with high levels of amino acids did not, however, show the increase in ODC activity observed in the regenerating rat liver (16,97) and phytohaemagglutinin-stimulated lymphocytes (50). The possibility exists that the larvae did not take up sufficient amino acids from the liquid culture media (no study of the enrichment of the amino acid pool was done), although this seems unlikely due to the observations that the larvae did take up significant quantities of polyamines and labeled uridine, thymidine, and leucine. It is possible that ODC is not under this postulated post-transcriptional control mechanism during early growth and development. None of the embryonic tissues in which ODC activity has been observed (13) has yet been studied with respect to the effect of increased levels of amino acids.

ODC activity was very high in 24-hour larvae and fell to a third or a quarter of the peak activity in 96-hour larvae (Table XI). SAMD activity assayed without added putrescine was generally high in the early stages of development (24-48 hours) and was decreased by one-half in the later stages (72-96 hours). Putrescine-stimulated SAMD, however, exhibited a much greater difference (5-6 fold) in activity between the highest level observed in 48-hour larvae and the levels observed in the older larvae.

Dion (7) has shown that the spermidine levels were highest in the young larvae of Drosophila and were reduced 6-7 fold between 24-96 hours of development. The concentration of putrescine was maintained at a low level throughout larval development. The fact that both ODC and SAMD activity were high during early development and reached their lowest activity in the older larvae suggest that the synthesis of putrescine and spermidine are coordinately controlled. Since there was no real increase in the putrescine concentration (7) during times of high ODC activity (24-48 hours), the size of the putrescine pool was probably closely controlled with most of the decarboxylated ornithine (putrescine) being used in spermidine synthesis.

Unlike the enzymes found in other tissues (see Literature Review), Mitchell and Rusch (56) observed that SAMD from P. polycephalum was not stimulated by putrescine at any stage of its development. SAMD from Drosophila larvae, however, does seem to possess a differential ability to be stimulated by putrescine. The enzyme activity in 24-hour larvae was only slightly increased by 2.0 mM putrescine (Table XI). This might indicate that, in the early stages of development, maximum rates of spermidine synthesis can be attained at the low levels of putrescine present during these stages of development (7,35). The maintenance of high cellular levels of spermidine could be a significant factor in the rapid synthesis of RNA during early development.

C. Thermolability and Cesium Chloride Extraction of RNA

The 28S species of rRNA found in D. melanogaster larvae was shown to be converted into "18S" species of RNA upon brief heat treatment in a manner similar to RNA isolated from other insect tissues (60,61,63-65). The possibility that a nuclease was introducing a specific break in the primary structure of the 28S rRNA during extraction is not likely due to the fact that RNA from chick polyribosomes co-extracted with RNA from Drosophila larvae does not become thermolabile. Since CsCl-extracted larval RNA was also found to be heat-dissociable, it is unlikely that phenol was causing a scission in the polynucleotide chain. The possibility still remains, however, that a ribosome-bound ribonuclease is cleaving the nucleotide bond at some point near the middle of the 28S rRNA nucleotide chain (61).

The "18S" products could not be separated from each other or from native 18S rRNA by acrylamide-gel electrophoresis or sucrose-gradient centrifugation in this investigation or other studies (60-65). Granboulan and Scherrer (112) have determined through electron microscope studies of denatured RNA that the two "18S" products of heated 28S rRNA from Bombyx mori have identical chain lengths equal to one-half the length of the unheated 28S rRNA. It appears likely that the scission occurs in a nuclease-sensitive region at the center of the molecule. If the larger ribosomal RNA species from insects is similar to the other eucaryotic rRNA's studied and consists of a series of double-helical segments

connected by short single-strand regions (113,114), some degree of symmetry in the folding of the polynucleotide chain may be implied. Sequence studies of yeast (115) and E. coli (116) rRNA also suggest that the larger species of rRNA may be composed of two identical or similar polynucleotide chains arising from a gene-doubling mechanism.

The CsCl-method of extracting RNA appears to offer several advantages over the phenol procedure most commonly used. CsCl-extraction yields RNA relatively free from protein with an absorbance spectrum similar to that obtained with phenol-extracted RNA (Fig. 13). The RNA can be isolated in one step and is separated from DNA without the use of DNase. By using a specially made rotor and a small CsCl gradient, RNA could be quantitatively extracted from small amounts of biological material (i.e., from isolated cells, nuclei, or chromosomes).

Some preliminary work has been done in attempting to reverse the aggregation of the rRNA following CsCl-extraction. Mild heat treatment (55^{o} for 45 seconds) of CsCl-isolated chick polyribosome RNA restored the normal 2:1 ratio of 28S/18S RNA without apparent loss due to degradation. Brief heating of RNA preparations to eliminate aggregation prior to gel-acrylamide electrophoresis is done routinely with RNA isolated from some sources (89). Incubation with DMSO has also partially reversed the aggregation of CsCl-extracted chick polyribosome RNA. However, heat and DMSO treatments of Drosophila CsCl-extracted RNA resulted in the dissociation of

the 28S rRNA. The CsCl procedure offers sufficient advantages to warrant further investigations into preventing or reversing the RNA aggregation during CsCl-extraction.

Purified 18S rRNA from chick polyribosomes and Drosophila larvae has been shown to aggregate in a CsCl gradient to form new distinct species of RNA as observed by acrylamide-gel electrophoresis and sucrose-gradient centrifugation (Fig. 18 and 19). The "27S" peak formed after passage of the phenol-extracted 18S rRNA through CsCl is probably composed of two aggregated 18S rRNA molecules. The RNA sedimenting slightly faster than this peak could result from the aggregation of three 18S rRNA molecules. It is possible that only a certain population of the 18S rRNA is able to aggregate in this manner. However, this was shown to be unlikely since the 18S RNA remaining after an initial pass through CsCl reaggregated to form larger molecules following a second passage through CsCl (Fig. 19). The amount of "aggregate" formed seems to be proportional to the concentration of RNA in the CsCl gradient. The relative increase in the size of the 28S peak which occurs when total RNA is CsCl-extracted from a tissue (or phenol-extracted total RNA is passed through CsCl) (Fig. 14-16) could be due to aggregation of two 18S rRNA molecules or interaction between an 18S and 28S rRNA molecule. The two peaks in Fig. 16 representative of species of RNA larger than 28S rRNA could be the result of multiple interactions of 18S rRNA with itself and/or 28S rRNA. These two peaks do, however, compare to the two species of RNA

sedimenting near the end of the gradient in the sample of 18S rRNA run through CsCl (Fig. 19).

The "18S" products from the heated 28S rRNA of Drosophila larvae aggregate in CsCl to form a 28S RNA indistinguishable from native 28S rRNA on acrylamide gels or sucrose gradients (Fig. 20 and 21). It has been postulated that the failure of other attempts to anneal the two halves of heated 26-28S rRNA from insects (61,63) is because only a short region of hydrogen bonding is involved in maintaining the stability of a specific helical region and preventing the separation of the two polynucleotide chains (61) (see Literature Review). The CsCl-extraction procedure must then provide a unique environment allowing the "18S" products to approach each other in the proper orientation to reform the bonds that hold the strands together. Further studies comparing the T_m' (temperature of 50% dissociation (61)) of native 28S rRNA and the reannealed "18S" products would provide information concerning the similarities of the bonds holding the polynucleotide chains together. The observations (data not shown) that mild heat treatment and DMSO can cause the reaggregated "18S" products to dissociate indicate that hydrogen bonds are involved in preventing strand separation in a manner similar to native 28S rRNA.

BIBLIOGRAPHY

1. Tabor, H. and Tabor, C.W., Spermidine, Spermine, and Related Amines (1964) Pharmacol. Revs. 16, 245-300
2. Herbst, E.J. and Snell, E.E., Putrescine and Related Compounds as Growth Factors for Hemophilus parainfluenzae (1949) J. Biol. Chem. 181, 47-54
3. Kremzer, L., Polyamines: Introductory Remarks (1970) Fed. Proc. 29, 1560-1562
4. Herbst, E.J. and Bachrach, U., eds., Metabolism and Biological Functions of Polyamines (1970) Ann. N. Y. Acad. Sci. 171, 691-1009
5. Russell, D.H., ed. (1973) Polyamines in Normal and Neoplastic Growth, Raven, New York
6. Cohen, S.S. (1971) Introduction to the Polyamines, Raven, New York
7. Dion, A.S. (1968) Putrescine and Polyamines: Relation to Growth and Development in Drosophila melanogaster, Ph.D. Thesis, University of New Hampshire
8. Herbst, E.J. and Dion, A.S., Polyamine Changes During Development of Drosophila melanogaster (1970) Fed. Proc. 29, 1563-1567
9. Heby, O., Polyamine Variations During Postembryonic Development of the Blowfly, Calliphora erythrocephala (1972) Insect Biochem. 2, 13-22
10. Caldarera, C.M. and Moruzzi, G., Polyamines and Nucleic Acid Metabolism in the Chick Embryo (1970) Ann. N. Y. Acad. Sci. 171, 709-722

11. Snyder, S.H., Kreuz, D.S., Medina, V.J. and Russell, D.H., Polyamine Synthesis and Turnover in Rapidly Growing Tissues (1970) Ann. N. Y. Acad. Sci. 171, 749-771

12. Russell, D.H. and Lombardini, J.B., Polyamines: (1) Enhanced S-Adenosyl-L-Methionine Decarboxylase in Rapid Growth Systems and (2) the Relationships Between Polyamine Concentrations and RNA Accumulation (1971) Biochim. Biophys. Acta 240, 273-286

13. Russell, D.H. and McVicker, T.A., Polyamines in the Developing Rat and in Supportive Tissues (1972) Biochim. Biophys. Acta 259, 247-258

14. Russell, D.H., Discussion: Putrescine and Spermidine Biosynthesis in Growth and Development (1970) Ann. N. Y. Acad. Sci. 171, 772-782

15. Hannonen, P., Raina, A. and Jänne, J., Polyamine Synthesis in the Regenerating Rat Liver: Stimulation of S-Adenosyl-L-Methionine Decarboxylase and Spermidine and Spermine Synthetases after Partial Hepatectomy (1972) Biochim. Biophys. Acta 273, 84-90

16. Fausto, N., Studies on Ornithine Decarboxylase in Normal and Regenerating Livers (1969) Biochim. Biophys. Acta 190, 193-201

17. Russell, D.H. and Snyder, S.H., Amine Synthesis in Rapidly Growting Tissues: Ornithine Decarboxylase Activity in Regenerating Rat Liver, Chick Embryo, and Various Tumors (1968) Proc. Natl. Acad. Sci. 60, 1420-1427

18. Brandt, J.T., Pierce, D.A. and Fausto, N., Ornithine Decarboxylase and Polyamine Synthesis During Kidney Hypertrophy (1972) Biochim. Biophys. Acta 279, 184-193

19. Jänne, J., Raina, A. and Siimes, M., Mechanism of the Stimulation of Polyamine Synthesis by Growth Hormone in Rat Liver (1968) Biochim. Biophys. Acta 166, 419-426

20. Panko, W.B. and Kenney, F.T., Hormonal Stimulation of Hepatic Ornithine Decarboxylase (1971) Biochem. Biophys. Res. Commun. 43, 346-350

21. Russell, D.H. and Taylor, R.L., Polyamine Synthesis and Accumulation in the Castrated Rat Uterus after Estradiol-17β Stimulation (1971) Endocrinology 88, 1397-1403

22. Kaye, A.M., Icekson, I. and Lindner, H.R., Stimulation by Estrogens of Ornithine and S-Adenosyl-L-Methionine Decarbosylase in the Immature Rat Uterus (1971) Biochim. Biophys. Acta 252, 150-159

23. Seiler, N. and Askar, A., Die Stimulierung des Leberwachstums und der Polyamin--und Nucleinsäurevermehrung durch Perfluorvaleriansaure (1972) Hoppe-Seyler's Z. Physiol. Chemie 353, 623-633

24. Wyatt, G.R., Rothaus, K., Lawler, D. and Herbst, E.J., Ornithine Decarboxylase and Polyamines in Silkmoth Pupal Tissues: Effects of Ecdysone and Injury (1973) Biochim. Biophys. Acta 304, 482-494

25. Russell, D.H., Polyamines in Growth--Normal and Neoplastic (1973) in Polyamines in Normal and Neoplastic Growth (Russell, D.H., ed.) pp. 1-13, Raven, New York

26. Goldstein, J., The Effect of Spermine on the Accumulation of Nucleic Acids and Protein in Mammalian Cells (1965) Exptl. Cell Research 37, 494-496

27. Barros, C. and Giudice, G., Effects of Polyamines on Ribosomal RNA Synthesis During Sea Urchin Development (1968) Exptl. Cell Research 50, 671-674

28. Barbiroli, B., Corti, A. and Caldarera, C.M., The Pattern of Ribonucleic Acid Species under Action of Spermine in the Chick Embryo (1971) Biochem. J. 123, 123-124

29. Caldarera, E.M., Casti, A., DeSanctis, B. and Moruzzi, G., Effect of Spermine on Free Nucleotide Turnover in Chick Embryo (1971) FEBS Lett. 14, 29-32

30. Raina, A. and Jänne, J., Polyamines and the Accumulation of RNA in Mammalian Systems (1970) Fed. Proc. 29, 1568-1574

31. Raina, A. and Jänne, J., Do Polyamines Play a Role in the Regulation of Nucleic Acid Metabolism? (1970) in Biogenic Amines Physiol. Regul. Symp. (Blum, J.J., ed.) pp. 275-300, Prentice-Hall, Englewood Cliffs

32. Fausto, B., RNA Metabolism in Isolated Perfused Normal and Regenerating Livers: Polyamine Effects (1972) Biochim. Biophys. Acta 281, 543-553

33. Wylie, C.C. and Russell, D.H., The Stimulation of RNA Synthesis in Mature Amphibian Oocytes by the Microinjection of Putrescine (1973) in Polyamines in Normal and Neoplastic Growth (Russell, D.H., ed.) pp. 289-298, Raven, New York

34. Gfeller, E., Levy, C.C. and Russell, D.H., Structural and Biochemical Changes in the Nucleolus in Response to Polyamines (1973) in Polyamines in Normal and Neoplastic Growth (Russell, D.H., ed.) pp. 335-342, Raven, New York

35. Dion, A.S. and Herbst, E.J., The Localization of Spermidine in the Salivary Gland Cells of Drosophila melanogaster and its Effect on ^{3}H-Uridine Incorporation (1967) Proc. Natl. Acad. Sci. 58, 2367-2371

36. Herbst, E.J., Byus, C.V. and Nuss, D.L., The Stimulation of RNA Synthesis by Spermidine: Studies with Drosophila Larvae and RNA Polymerase (1973) in Polyamines in Normal and Neoplastic Growth (Russell, D.H., ed.) pp. 71-90, Raven, New York

37. Nuss, D.L., Polyamine and Potassium Chloride Mediated Stimulation of in vitro Transcription (1973) Ph.D. Thesis, University of New Hampshire

38. Roeder, R.G. and Rutter, W.J., Specific Nucleolar and Nucleoplasmic RNA Polymerases (1970) Proc. Natl. Acad. Sci. 65, 675-682

39. Barbiroli, B., Corti, A. and Caldarera, C.M., RNA Polymerase Activity in Purified Nuclei from Rat Prostate Gland in the Presence of Polyamines (1971) FEBS Lett. 19 169-172

40. Jänne, J. and Raina, A., On the Stimulation of Ornithine Decarboxylase and RNA Polymerase Activity in Rat Liver after Treatment with Growth Hormone (1969) Biochim. Biophys. Acta 174, 769-772

41. Russell, D.H., Levy, C.C. and Taylor, R.L., Increased DNA Associated with Rat Liver Nucleoli when Isolated with Spermidine (1972) Biochem. Biophys. Res. Commun. 47, 212-217

42. Erdmann, V.A., Thomas, G.A., Norton, J.W. and Herbst, E.J., The Effect of Polyamine on the Enzymatic Degradation of Ribosomes (1968) Biochim. Biophys. Acta 157, 43-51

43. Brewer, E.N., Inhibition of Alkaline Ribonuclease Activity by Polyamines (1972) Exptl. Cell Research 72, 586-588

44. Khawaja, J.A., Increased Interaction of Ribosomes and Ribosomal Subparticles with Endoplasmic Reticulum Membranes in vitro: Effect of Spermine and Magnesium Ions (1971) Biochim. Biophys. Acta 254, 117-128

45. Bagni, N., Corsini, E. and Fracasini, D.S., Growth Factors and Nucleic Acid Synthesis in Helianthus tuberosus I. Reversal of Actinomycin D Inhibition by Spermidine (1971) Physiol. Plant. 24, 112-117

46. Jänne, J. and Williams-Ashman, H.G., On the Purification of L-Ornithine Decarboxylase from Rat Prostate and Effects of Thiol Compounds on the Enzyme (1971) J. Biol. Chem. 246, 1725-1732

47. Raina, A. and Jänne, J., Biosynthesis of Putrescine: Characterization of Ornithine Decarboxylase from Regenerating Rat Liver (1968) Acta Chem. Scand. 22, 2375-2378

48. Rahaila, E.L., Kekomäki, M., Jänne, J., Raina, A. and Räittä, N.C.R., Inhibition of Pyridoxal Enzymes by L-Canaline (1971) Biochim. Biophys. Acta 227, 337-343

49. Beck, W.T., Bellantone, R.A. and Cannellakis, E.S., Puromycin Stimulation of Rat Liver Ornithine Decarboxylase Activity (1973) Nature 241, 275-277

50. Kay, J.E., Lindsay, V.J. and Cooke, A., Ornithine Decarboxylase in Phytohaemagglutinin Stimulated Lymphocytes: Control of Degradation Rate by Amino Acids (1972) FEBS Lett. 21, 123-126

51. Jänne, J., Schenone, A. and Williams-Ashman, H.G., Separation of Two Proteins Required for the Synthesis of Spermidine from S-Adenosyl-L-Methionine and Putrescine in Rat Prostate (1971) Biochem. Biophys. Res. Commun. 42, 758-764

52. Jänne, J. and Williams-Ashman, H.G., Dissociation of Putrescine-Activated Decarboxylation of S-Adenosyl-L-Methionine from the Enzymatic Synthesis of Spermidine and Spermine by Purified Prostatic Enzyme Preparations (1971) Biochem. Biophys. Res. Commun. 42, 222-229

53. Hannonen, P., Jänne, J. and Raina, A., Separation and Partial Purification of S-Adenosyl-Methionine Decarboxylase and Spermidine and Spermine Sunthetases from Rat Liver (1972) Biochem. Biophys. Res. Commun. 46, 341-348

54. Feldman, M.J., Levy, C.C. and Russell, D.H., The Purification of S-Adenosyl-L-Methionine Decarboxylase from Rat Liver: Inability to Separate Decarboxylation from Spermidine Synthase (1971) Biochem. Biophys. Res. Commun. 44, 675-681

55. Feldman, M.J. and Russell, D.H., Purification and Characterization of S-Adenosyl-L-Methionine Decarboxylase from Rat Liver (1972) Biochemistry 11, 671-676

56. Mitchell, J.L.A. and Rusch, H.P., Regulation of Polyamine Synthesis in Physarum polycephalum During Growth and Differentiation (1973) Biochim. Biophys. Acta 297, 503-516

57. Schmidt, G.L.S. and Cantoni, G.L., Adenosylmethionine Decarboxylase in Developing Rat Brain (1973) J. Neurochem. 20, 1373-1385

58. Pegg, A.E., Corti, A. and Williams-Ashman, H.G., Paradoxical Enhancement of S-Adenosyl-Methionine Decarboxylase in Rat Tissues Following Administration of the Specific Inhibitor, Methyl-Glyoxal-Bis (Guanyl Hydrazone) (1973) Biochem. Biophys. Res. Commun. 52, 696-701

59. Fillingame, R.H. and Morris, D.R., S-Adenosyl-L-Methionine Decarboxylase During Lymphocyte Transformation: Decreased Degradation in the Presence of a Specific Inhibitor (1973) Biochem. Biophys. Res. Commun. 52, 1020-1025

60. Applebaum, S.W., Ebstein, R.P. and Wyatt, G.R., Dissociation of Ribosomal Ribonucleic Acid from Silkmoth Pupae by Heat and Dimethyl Sulfoxide: Evidence for Specific Cleavage Points (1966) J. Mol. Biol. 21, 29-41

61. Shine, J. and Dalgarno, L., Occurrence of Heat-Dissociable Ribosomal RNA in Insects: The Presence of Three Polynucleotide Chains in 26S RNA from Cultured Aedes aegypti Cells (1973) J. Mol. Biol. 75, 57-72

62. Stevens, A.R. and Pachler, R.F., Discontinuity of 26S rRNA in Acanthamoeba castellani (1972) J. Mol. Biol. 66, 225-237

63. Ishikawa, H. and Newburgh, R.W., Studies of the Thermal Conversion of 28S RNA of Galleria mellonella (L.) to an 18S Product (1972) J. Mol. Biol. 64, 135-144

64. Petri, W.H., Fristrom, J.W., Stewart, D.J. and Hanly, E.W., The in vitro Synthesis and Characteristics of Ribosomal RNA in Imaginal Discs of Drosophila melanogaster (1971) Mol. Gen. Genet. 110, 245-262

65. Greenberg, J.R., Synthesis and Properties of RNA in Drosophila (1969) J. Mol. Biol. 46, 85-98

66. Hunt, J.A., Terminal-Sequence Studies of High Molecular Weight Ribonucleic Acid (1965) Biochem. J. 95, 541

67. Pene, J.J., Knight, E.K. and Darnell, J.E., Characterization of New Low Molecular Weight RNA in HeLa Cell Ribosomes (1968) J. Mol. Biol. 33, 609-623

68. Sy, J. and McCarty, K.S., Characterization of 5.8-S RNA from a Complex with 26-S Ribosomal RNA from Arbacia punctulata (1970) Biochim. Biophys. Acta 199, 86-94

69. Pearl, R., Allen, A. and Penniman, W.B.D., Culture Media for Drosophila II. A New Synthetic Medium and its Influence on Fertility at Different Densities of Population (1926) Am. Naturalist 60, 357

70. Demerec, M., ed. (1965) Biology of Drosophila, Hafner, New York

71. Wang, T.Y., Isolation of Mammalian Nuclear RNA (1968) in Methods in Enzymology (Grossman, L. and Moldave, K., eds.) Vol. 12, pp. 115-120, Academic, New York

72. Millette, R.L. and Trotter, C.D., Initiation and Release of RNA by DNA-Dependent RNA Polymerase (1970) Proc. Natl. Acad. Sci. 66, 701-708

73. Birnboim, H.C., Optimal Conditions for Counting of Precipitated ^{3}H-RNA on Glass-Fiber Filters (1970) Anal. Biochem. 37, 178-182

74. Emerson, C.P., Regulation of the Synthesis and the Stability of Ribosomal RNA During Contact Inhibition of Growth (1971) Nature New Biol. 232, 101-106

75. Tsuboi, K.K. and Price, T.D., Isolation, Detection and Measure of Microgram Quantities of Labeled Tissue Nucleotides (1959) Arch. Biochem. Biophys. 81, 223-237

76. Silver, M.J., Rodalewicz, I., Douglas, Y. and Park, D., High Voltage Paper Electrophoresis of Nucleotides (1970) Anal. Biochem. 36, 525-527

77. Munro, H.N. and Fleck, A., The Determination of Nucleic Acids (1966) in Methods of Biochemical Analysis (Glick, D., ed.) Vol. 14, pp. 113-176, Interscience, New York

78. Church, R.B. and Robertson, F.W., A Biochemical Study of the Growth of Drosophila melanogaster (1966) J. Exptl. Biol. 162, 337-351

79. Cowgill, R.W. and Pardee, A.R. (1957) Experiments in Biochemical Research Techniques, John Wiley, New York

80. Weinberg, R.A., Loening, U., Willems, M. and Penman, S., Acrylamide Gel Electrophoresis of HeLa Cell Nucleolar RNA (1967) Proc. Natl. Acad. Sci. 58, 1088-1095

81. Loening, U.E., The Determination of the Molecular Weight of Ribonucleic Acid by Polyacrylamide-Gel Electrophoresis (1969) Biochem. J. 113, 131-138

82. Casola, L. and DeMatteo, G., Studies on the Dansylation Reaction by the Use of ^{14}C-Dansyl Chloride (1972) Anal. Biochem. 49, 416-420

83. Seiler, N., Use of the Dansyl Reaction in Biochemical Analysis (1970) in Methods of Biochemical Analysis (Glick, D., ed.) Vol. 18, pp. 259-337, Interscience, New York

84. Preparation of Samples for Liquid Scintillation Counting, Nuclear Chicago Corp., Des Plaines, Ill.

85. Girard, M., Latham, H., Penman, S. and Darnell, J.E., Entrance of Newly Formed Messenger RNA and Ribosomes into HeLa Cell Cytoplasm (1965) J. Mol. Biol. 11, 187-201

86. Fraenkel-Conrat, H., Singer, B. and Tsugita, A., Purification of Viral RNA by Means of Bentonite (1961) Virology 14, 54-58

87. Litt, M. and Ingram, V.M., Chemical Studies in Amino Acid Acceptor Ribonucleic Acids II. Attempts at Partial Digestion of Yeast Amino Acid Acceptor Ribonucleic Acid with Pancreatic Ribonuclease (1963) Biochemistry 3, 560-564

88. Weiner, S.L., Weiner, R., Urivetzky, M. and Meilmon, E., Inhibition of Ribonuclease by Diethyl Pyrocarbonate (1972) Biochim. Biophys. Acta 259, 378-385

89. Rubinstein, L. and Clever, U., Chromosome Activity and Cell Function in Polytenic Cells V. Developmental Changes in RNA Synthesis and Turnover (1972) Dev. Biol. 27, 519-537

90. Plagemann, P.G., Nucleotide Pools of Novikoff Rat Hepatoma Cells Growing in Suspension Culture (1970) J. Cell Physiol. 77, 213-240

91. Ritossa, F.M., Atwood, K.C. and Spiegleman, S., On the Redundancy of DNA Complementary to Amino Acid Transfer RNA and its Absence from the Nucleolar Organizer Region of Drosophila melanogaster (1966) Genetics 54, 663-676

92. Emerson, C.P. and Humphreys, T., A Simple and Sensitive Method for Quantitative Measurement of Cellular RNA Synthesis (1971) Anal. Biochem. 40, 254-266

93. Kijma, S. and Witt, F.H., Rate of Nuclear Ribonucleic Acid Turnover in Sea Urchin Embryos (1969) J. Mol. Biol. 40, 235-246

94. Weber, M.J., Ribosomal RNA Turnover in Contact Inhibited Cells (1972) Nature New Biol. 235, 58-60

95. McElhone, M.J., O'Connor, P.J. and Craig, A.W., The Stability of Rat Liver Ribonucleic Acid in vivo after Methylation with Methyl Methanesulfphonate or Dimethyl-nitrosoamine (1971) Biochem. J. 125, 821-827

96. Price, G.M., Protein Synthesis and Nucleic Acid Metabolism in the Fat Body of the Larvae of the Blowfly, Calliphora erthrocephala (1969) J. Insect Physiol. 15, 931-944

97. Fausto, N., The Control of Ornithine Decarboxylase Activity During Liver Regeneration (1971) Biochim. Biophys. Acta 238, 116-128

98. Pegg, A.E. and Williams-Ashman, H.G., On the Role of S-Adenosyl-L-Methionine in the Biosynthesis of Spermidine by Rat Prostate (1969) J. Biol. Chem. 241, 682-693

99. Jänne, J., Williams-Ashman, H.G. and Schenone, A., Spermidine Synthesizing Enzymes in Baker's Yeast (1971) Biochem. Biophys. Res. Commun. 43, 1362-1368

100. Manen, C.A. and Russell, D.H., Polyamines in Marine Invertebrates (1973) in Polyamines in Normal and Neoplastic Growth (Russell, D.H., ed.) pp. 277-288, Raven, New York

101. Raina, A., Siimes, M. and Jänne, A., Effect of Spermidine on RNA Synthesis in Erhlich Ascites Cells (1968) Scand. J. Clin. Lab. Invest. 21, 10-19

102. Otsuka, H., Toxic Effects of Spermidine on Normal and Transformed Cells (1971) J. Cell Science 9, 71-84

103. Bachrach, U., Inactivation of Myxoviruses by Oxidized Polyamines (1971) J. Gen. Virol. 11, 1-9

104. Weinmann, R., Regulation of Ribosomal RNA and 5S RNA Synthesis in Drosophila melanogaster: I. Bobbed Mutants (1972) Genetics 72, 267-276

105. Darnell, J.E., Ribonucleic Acids from Animal Cells (1968) Bacteriol. Revs. 32, 262-290

106. Vaughan, M., Soeiro, R., Warner, J. and Darnell, J., The Effects of Methionine Deprivation on Ribosome Synthesis in HeLa Cells (1967) Proc. Natl. Acad. Sci. 58, 1527-1534

107. Willems, M., Penman, M. and Penman, S., The Regulation of RNA Synthesis and Processing in the Nucleolus During Inhibition of Protein Synthesis (1969) J. Cell Biol. 41, 177-194

108. Papaconstantinou, J. and Julku, E.M., The Regulation of Ribosomal RNA Synthesis and Ribosome Assembly in the Vertebrate Lens (1968) J. Cell Physiol. 72, 161-180

109. Mohan, J. and Ritossa, F.M., Regulation of Ribosomal RNA Synthesis and its Bearing on the Bobbed Phenotype in Drosophila melanogaster (1970) Dev. Biol. 22, 259-512

110. Egyhazi, E. and Edstrom, J.E., Evidence for the Transport of 4S RNA from the Nucleus to the Cytoplasm in Salivary Gland Cells of Chironomus tentans (1972) Biochem. Biophys. Res. Commun. 46, 1551-1556

111. Burdon, R.H., Ribonucleic Acid Maturation in Animal Cells (1971) Prog. Nucl. Acid Res. 11, 33-73

112. Granboulan, N. and Scherrer, K., Visualization in the Electron Microscope and Size of RNA from Animal Cells (1969) Eur. J. Biochem. 9, 1-20

113. Cox, R.A., A Spectrophotometric Study of the Secondary Structure of Ribonucleic Acid Isolated from the Smaller

and Larger Ribosomal Subparticles of Rabbit Reticulocytes (1970) Biochem. J. 117, 101-118

114. Attardi, G. and Amaldi, F., Structure and Synthesis of Ribosomal RNA (1970) Ann. Rev. Biochem. 39, 183-226

115. Van Den Bos, R.C. and Planta, R.J., Structural Comparison of 26S and 17S Ribosomal RNA of Yeast (1970) Nature 225, 183-184

116. Fellner, P. and Sanger, F., Sequence Analysis of Specific Areas of the 16S and 23S Ribosomal RNAs (1968) Nature 219, 236-238