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A Study of 5-Fluorouracil: Its Effects and Mode of Action in *Drosophila melanogaster*

John B. Jenkins Jr.
Utah State University

A STUDY OF 5-FLUOROURACIL: ITS EFFECTS AND MODE OF ACTION IN *DROSOPHILA MELANOGASTER*

by

John B. Jenkins, Jr.

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Approved:

_____
Major Professor

_____
Head of Department

_____
Dean of Graduate Studies

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INTRODUCTION

Investigations involving the fluorinated pyrimidine, 5-fluorouracil, and its effects on viruses, bacteria, plants and mammals, have brought to light the following facts: FU[1] inhibits the growth of bacteria, plants and mammalian cells and it decreases the ability of certain viruses to produce progeny. In other words, FU is an antimetabolite that inhibits growth and/or reproduction. Two mechanisms have emerged from these studies to define the mode of action of FU. The first mechanism involves the incorporation of the compound into the RNA of the organism thus altering the base sequence and creating a non functional or damaging nucleic acid. The second mechanism concerns the blockage of the methylating enzyme, thymidylate synthetase, which catalyzes the reaction converting dUMP to TMP. When this enzyme is blocked it would, of course, follow that DNA synthesis would be inhibited. FU has not been shown to be

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[1]The following abbreviations will be used throughout this thesis.
FU - fluorouracil
FUdR - fluorodeoxyuridine
FUdRP - fluorodeoxyuridylic acid
FURP - fluorouridylic acid
TdR - thymidine
TMP - thymidylic acid
UR - uridine
dUMP - deoxyuridylic acid
DNA - deoxyribose nucleic acid
RNA - ribose nucleic acid
AHAI - alkaline hydrolyzed acid-insoluble
AHAS - alkaline hydrolyzed acid-soluble

incorporated into DNA but 5-trifluoromethyluracil has (Gottschling and Heidelberger, 1963).

No work has been done with FU and its effects on Drosophila but some work has been done with its biological effects on the housefly, Musca domestica (Kilgore and Painter, 1962). In Musca, FU was very toxic when fed in the diet. When 5-FU-2-^{14}C was ingested, it was found that almost all of the compound was passed out in the excreta as waste product. Some ^{14}C, however, was incorporated into the egg, either as FU or one of its metabolic products. An inverse relationship existed between the amount of ^{14}C in the egg and hatchability. To account for the lethality it has been proposed (Kilgore, personal communication) that the FU is incorporated into the nucleic acids, specifically RNA, producing a non functional nucleic acid.

In preliminary investigations with FU and its biological effects on Drosophila, it was noted that the compound had a pronounced lethal effect. As was noted earlier, two main possibilities exist to explain this lethal effect in Drosophila and it is the purpose of this investigation to examine those possibilities.

REVIEW OF LITERATURE

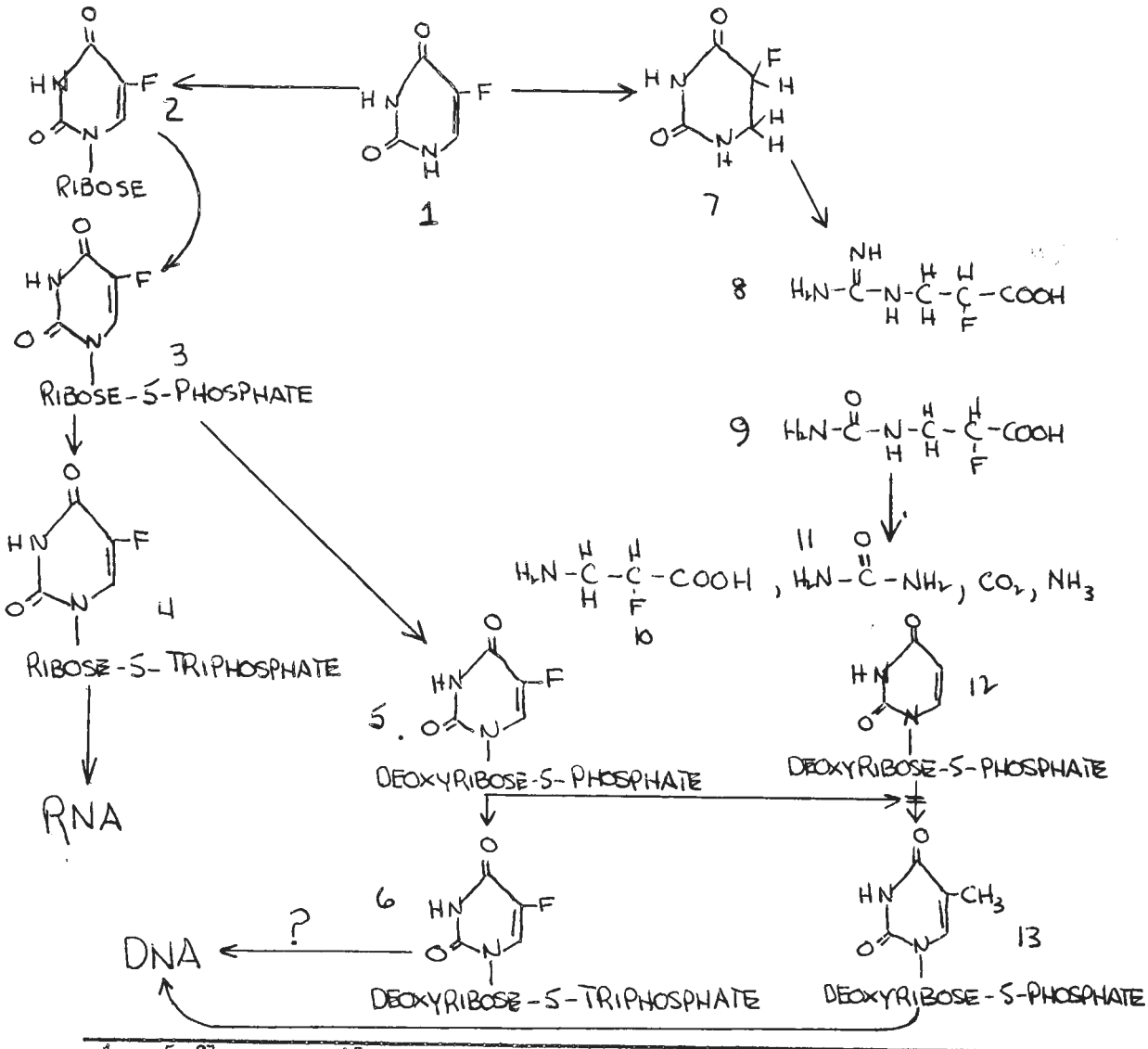
Aside from the normal purine and pyrimidine bases, such as adenine, guanine, cytosine, uracil, methyl cytosine, and hydroxymethyl cytosine, that are incorporated into DNA and RNA, some abnormal base analogs can also be incorporated when they are present in the environment of the nucleic acids during replication. Some of these analogs are mutagenic when incorporated into the nucleic acids because they alter the normal base sequence by inducing base pairing mistakes. We shall concern ourselves here with only the halogenated pyrimidines, 5-iodouracil, 5-bromouracil, 5-chlorouracil, and 5-fluorouracil, with all emphasis on the latter. The first three can replace thymine in DNA. These three thymine analogs are not inhibitory to any great extent but are primarily mutagenic. Their mutagenic effect has been attributed to base pairing mistakes that occur as a consequence of their incorporation into DNA (Taylor, 1963).

FU has been recently synthesized (Heidelberger, et. at., 1957) because it appeared plausible that replacement of a hydrogen atom with a fluorine atom in the pyrimidine ring should lead to an analog of considerable potency. This belief was based on the profound biological effects often exhibited when fluorine was substituted for hydrogen in several unrelated classes of compounds: The high toxicity of fluoroacetate (Buffa and Peters, 1949), the amino acid inhibitory properties of p-fluorophenylalanine (Armstrong and Lewis, 1951), and

the increased biological potency of fluorinated steroids (Fried and Sabo, 1954) serve as illustrations of this point.

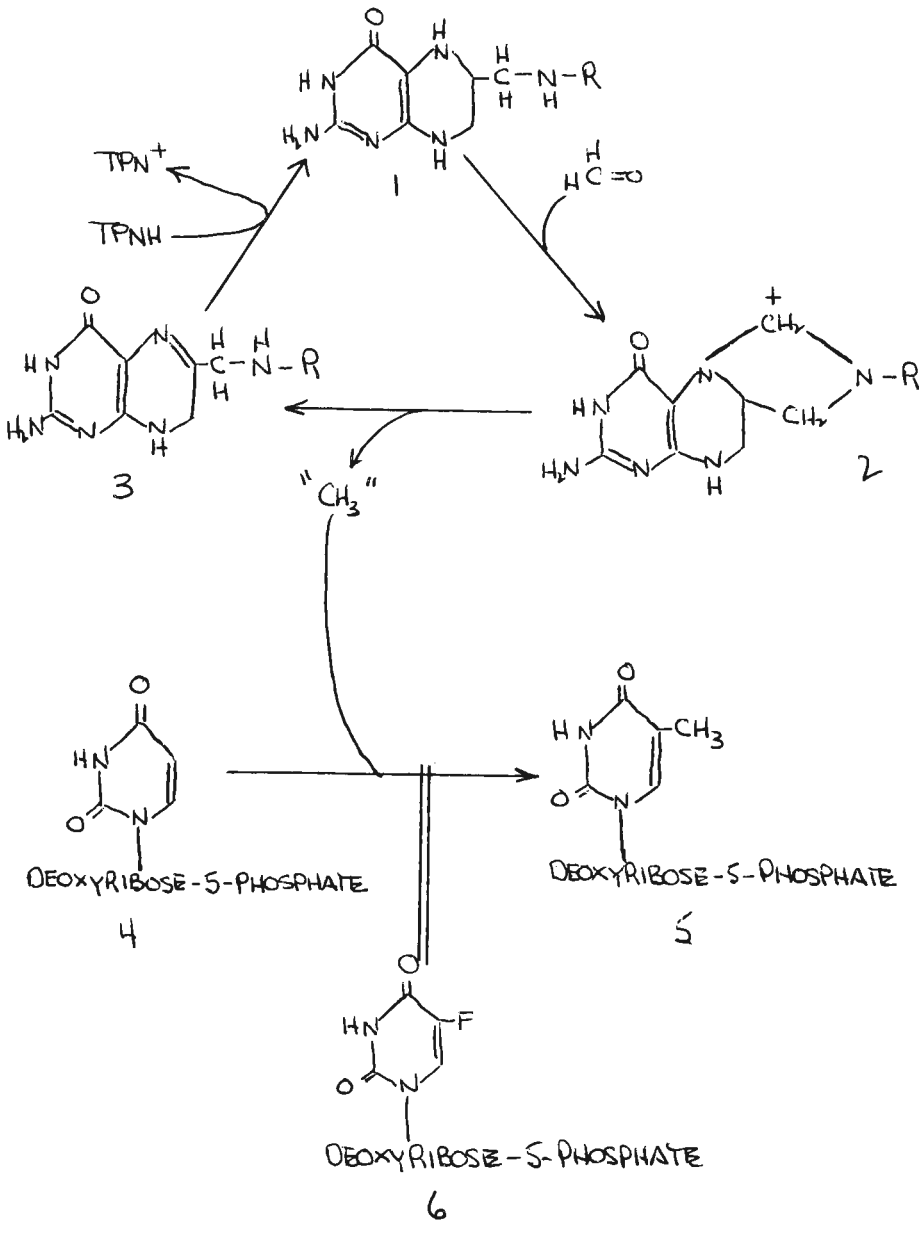
It was believed that FU would manifest tumor-inhibitory properties in mammals, and indeed it did (Heidelberger et al., 1957). Since this initial study by Heidelberger (1957), FU has been used extensively in cancer, viral, and bacterial research, and less extensively in plant and non mammalian animal research. The most prevalent characteristic displayed by the compound in practically all organisms studied was its marked inhibitory effect on cellular and sub-cellular growth.

Previous experimentation with mammalian cells and bacteria has clearly demonstrated that the inhibition of the methylating enzyme, thymidylate synthetase, and, hence, of DNA biosynthesis, was the primary mechanism by which FU inhibited the growth of those cells (Bosch et al., 1958; Harbers et al., 1959; Hartmann and Heidelberger, 1961). The actual inhibitor of thymidylate synthetase has been shown to be FUdRP (Cohen et al., 1958; Hartmann and Heidelberger, 1961), and, further, the formation of this inhibitory nucleotide from FU has been found to follow the same metabolic pathway as that of dUMP from uracil (Chaudhuri et al., 1958; Harbers et al, 1959; Skold, 1960). Figure 1 has been constructed from various sources to demonstrate the possible metabolic pathways which FU may traverse, and Figure 2 shows the mechanism by which the methylating enzyme, thymidylate synthetase, converts dUMP into TMP and the step blocked by FUdRP (Birnie et al., 1963; Harbers et al., 1959; Heidelberger et al., 1958; Mukherjee and Heidelberger, 1960).



1 - 5-fluorouracil
2 - 5-fluorouridine
3 - 5-fluorouridine-5'monophosphate
4 - 5-fluorouridine-5'triphosphate
5 - 5-fluorodeoxyuridine-5'-monophosphate
6 - 5-fluorodeoxyuridine-5'-triphosphate
7 - 4,5-dihydro-5-fluorouracil
8 - alpha-fluoro-beta-guanidoproprionic acid
9 - alpha-fluoro-beta-ureidoproprionic acid
10 - alpha-fluoro-beta-alanine
11 - urea
12 - deoxyuridine-5'-monophosphate
13 - thymidine-5'-monophosphate

Figure 1. Metabolism of 5-fluorouracil



1 - tetrahydrofolic acid
2 - 5,10-methylene-tetrahydrofolic acid
3 - dihydrofolic acid
4 - deoxyuridine-5'-monophosphate
5 - thymidine-5'-monophosphate
6 - 5-fluorodeoxyuridine-5'-monophosphate

Figure 2. Mechanism of the conversion of dUMP to TMP and probable mode of action of FUdRP

A second mechanism which was also operative in the inhibition of cellular and sub-cellular growth was the incorporation of FU into RNA thus creating a non functional or damaging nucleic acid (Chaudhuri et al., 1958; Harbers et al., 1959; Horowitz and Chargaff, 1959). This latter mechanism was probably more important in the diptera (Kilgore and Painter, 1962; Kilgore, personal communication).

The response to FU by bacteria and viruses was not at all consistent. It has been found that FU was inhibitory but the degree and type of inhibition varies extensively (Cohen et al., 1958; Goodman et al., 1960; Heidelberger et al., 1957; Saukkonen et al., 1960). This variability was evidenced in some cases by the ability of an exogenous source of thymine to bypass the metabolic block of thymidylate synthetase and relieve the inhibition; in other cases an exogenous source of uracil had the ability to partially relieve the inhibition (Reich and Mandel, 1964), and in still other cases, neither thymine nor uracil could relieve the inhibition (Davern and Bonner, 1958).

In Tobacco Mosaic Virus, 28 to 47% of the uracil in RNA was replaced by FU (Gordon and Staehelin, 1959). The consequences of this massive incorporation of FU were not as drastic as one would perhaps have visualized. The progeny of the substituted virus were normal but the ability of the substituted virus to induce progeny synthesis in the host cell was reduced.

FU has also been shown to be incorporated into the RNA of Escherichia coli (Horowitz and Chargaff, 1959; Horowitz et al., 1958;, Musca domestica (Kilgore, personal communication), and mammalian cells (Harbers et al., 1958; Chaudhuri et al., 1958).

In the plant kingdom, Vicia faba and Arabidopsis thaliana have been studied with respect to FUdR. In Vicia, FUdR seems to induce breaks in the chromosomes but the precise mechanism is in dispute. On one side of the dispute it was believed that FUdR, by inhibiting DNA synthesis, produced lesions in the chromosomes, and hence, led to chromosomal fragmentation when the chromosomes moved apart at anaphase. To further support this side of the dispute, treatment of these lesions with an exogenous source of TdR seemed to cure the lesions. X-Ray induced lesions failed to repair in the presence of FUdR because it was postulated that the nucleotide inhibited DNA biosynthesis and hence blocked repair of the lesions (Taylor et al., 1962).

On the other side of the dispute it was postulated that FUdR produced lesions independently of DNA synthesis. To support this, evidence was presented showing that the breaks occurred in the chromosomes when the cell was not undergoing DNA synthesis. To explain the X-ray data that was obtained by Taylor, it was postulated that somehow the FUdR sensitized the chromosome to the breakage effect of X-rays. It was also proposed that FUdR competes with TdR for sites of chromosome breakage (Bell and Wolff, 1964).

In Arabidopsis, FU inhibited growth but the inhibition was overcome by the addition of exogenous thymine (Brown, 1962; Brown and Smith, 1964).

A relatively small amount of work has been done with FU and its effects on diptera. Of the work that has been done, most of it was concerned with the housefly. No work has been reported with Drosophila.

Studies with FU and Musca have shown that the compound caused temporary rather than permanent sterilization when it was fed at low levels in the diet to the adult insects immediately preceding oviposition (Painter and Kilgore, 1964). Most of the compound was excreted rapidly as waste material, although a very small amount was incorporated into the eggs, either as FU or a metabolic product. By utilizing FU-2-^{14}C (Kilgore and Painter, 1962), it has been shown that there was a correlation between the amount of ^{14}C incorporated into the egg and egg viability. In this paper, however, it wasn't determined if the undegraded FU molecules or some of their metabolic products were the source of the radioactivity. The largest quantity of label was found in eggs deposited during the first day of oviposition. On each day following the start of oviposition the amount of ^{14}C found in the egg decreased until very little was present in eggs laid after the fourth day.

The RNA of the housefly eggs was extracted with $HClO_4$ and examined for the presence of ^{14}C label (Kilgore and Painter, 1964; Kilgore, personal communication). It was found that approximately 80% of the total amount of label incorporated into the eggs was acid-insoluble, while about 20% was acid-soluble. The nucleic acids would be found in the acid-insoluble fraction. Chromatograms of the AHAS fraction showed the presence of FU-2-^{14}C and FURP-1-^{14}C along with a spot that couldn't be identified. Chromatograms of the AHAI fraction showed only the presence of FURP-2-^{14}C. Purified egg RNA was also prepared, hydrolyzed with KOH and analyzed by chromatography (Levenbrook et al., 1958). The results showed only the presence of

FURP-2-^{14}C. The amount of FU actually incorporated into the RNA of the Musca egg was very small when compared to other biological systems.

It has also been reported that FU sterilizes the female but not the male housefly (Crystal, 1963).

These studies, then, indicate that FU may sterilize the housefly eggs because it replaced the normal metabolite, uracil, in RNA. It may also be incorporated into DNA, but based on previous investigations this seems unlikely. It was apparent that in Musca the FU was transferred from the diet to the fertilized egg through the female.

Except for these studies with Musca, there have been no reports on the mechanism of action or biological effects of FU in the dipterans. Therefore, in a consideration of the possible mechanisms by which FU may exert its inhibitory properties in Drosophila, the two most likely possibilities seem to require either the incorporation of the compound into RNA, thus creating a non functional nucleic acid, or the blockage of the methylating enzyme, thymidylate synthetase, necessary for the conversion of dUMP into TMP.

Other explanations may account for the inhibitory properties of FU in Drosophila. The metabolic products of FU, such as alpha-fluoro-beta-guanidopropionic acid, alpha-fluoro-beta-ureidoproprionic acid, and alpha-fluoro-beta-alanine, may be toxic to the organism. These possibilities haven't been explored yet and will not be explored in this investigation.

METHODS AND MATERIALS

Stocks Utilized in the Experiments

The tumorous head stock (tu-h) was selected for experimentation because of its sensitivity to environmental alterations (Gardner and Ratty, 1952). This stock has been maintained in the laboratories of the University of Utah and Utah State University since 1946 and has undergone intensive investigation since that time under the direction of Dr. Eldon J. Gardner and associates. Samarkand was selected as the wild-type stock for comparison purposes. These stocks were maintained on a medium of the following composition:

corn meal	200 gms
agar	30 gms
molasses	338 gms
water	3750 mls
moldex solution	35 mls
Fleischmann's yeast	--

The moldex solution was prepared by adding 1 gm of methyl-p-hydroxy-benzoate to 10 mls of 70% ethanol. The medium was autoclaved in half-pint milk bottles for 20 minutes at 15 pounds pressure. The yeast was sprinkled on the cooled media. The stocks were maintained at 25°C as were all experiments.

Experimentation to Determine the Effects of Fluorouracil on Eggs

Egg collection technique

About 150 male and 150 female tu-h flies were placed in a laying cage (King, 1955) which was constructed by cutting $2\frac{1}{2}$ inch plastic pipe into cylinders about a quarter of an inch in heighth and then covering the open ends with dacron netting. The laying cage containing the flies was placed in a petri dish, the bottom of which was covered with a cloth, moistened with a saturated sucrose solution and seeded with powdered Fleischmann's dry yeast. The cages were transferred at regular intervals. When the eggs were ready for collection, they were washed onto a Buchner funnel, the bottom of which was covered with a moist cloth. The cloth retained the eggs but allowed the yeast cells to pass through. The funnel was part of an aspirating system and a gentle suction was applied as the eggs were washed.

Egg dechorionation

The eggs thus collected were washed into a dilute sodium hypochlorite solution (5 mls of commercial "Chlorox" per 100 mls of Ringers) where they remained for 20 minutes. At the end of the 20 minutes the eggs were washed back onto the Buchner funnel and thoroughly rinsed.

Egg experimentation

Dechorionated, washed, and rinsed tu-h eggs at 2 hours $\pm$ 2 hours post-lay were washed into beakers containing one of the following solutions:

1) Insect Ringers

2) Insect Ringers + 0.02 mg/ml FU

3) Insect Ringers + 0.05 mg/ml FU

The eggs were retained in the solutions for 6 hours then washed and placed on a cloth soaked in saturated sucrose solution and seeded with powdered Fleischmann's dry yeast.

Feeding Experimentation with Larvae to Determine the Effects of Fluorouracil

Larvae collection technique

Eggs deposited on the cloth were allowed to hatch. The age of the larvae was regulated by collecting the larvae at specific intervals. The larvae were collected by touching the sides of the organism with a blunt teasing needle and picking it off the medium. The larvae thus collected were washed onto the Buchner funnel and rinsed.

Feeding experimentation with larvae

Feeding experiments with FU were set up for two reasons: 1) to determine the effects of the compound on larvae, and 2) to determine if the effects can be reversed by exogenous sources of TdR and UR. The basic medium used in all feeding experiments was composed of:

sucrose	25 gms
brewers yeast	25 gms
agar	2, 5 gms
tartaric acid	1 gm
water	240 mls

2.5 gms of this medium were used per shell vial and 50 larvae were

placed in each vial. Samarkand and tu-h were tested for their response to the following experimental conditions:

1) 0.01 mg FU/gm medium
2) 0.064 mg FU/gm medium
3) 0.12 mg FU/gm medium
4) 2.4 mg TdR/gm medium
5) 12.0 mg TdR/gm medium
6) 2.4 mg UR/gm medium
7) 0.12 mg FU + 2.4 mg TdR/gm medium
8) 0.12 mg FU + 12.0 mg/TdR/gm medium
9) 0.12 mg FU + 2.4 mg UR/gm medium
10) 0.12 mg FU + 2.4 mg UR + 2.4 mg TdR/gm medium
11) Control (no additive to the basic medium)

All larvae were first instar. Observations were made on each vial every 24 hours and the pupae formed between each observation were recorded. In some cases, the experiment was terminated after 168 hours post-hatch because of the unhealthy condition of the larvae. The studies to determine the effect of FU alone were carried out to 264 hours post-hatch.

Injection Experimentation with Larvae and Adults to Determine Fluorouracil Effects

Preparation of injection apparatus

Pasteur pipettes were drawn out over a small flame until the tip diameter was equivalent to that of a hair. The tip was then ground to a beveled point using as a grinding surface a small metal disc covered

with wetted emory paper. The disc was connected to a small electric motor.

Larvae 72 hours ± 4 hours post-hatch were placed on Scotch Brand Drafting Tape No. 23 to hold them in place. The larvae were injected in the posterior-dorsal third of the body then floated off the tape with water. The injected larvae were placed on a cloth soaked in a saturated sucrose solution and seeded with powdered Fleischmann's dry yeast.

The adult Drosophila were held down, without the aid of an adhesive surface, by a soft camel's hair brush. The injections were made into the ventral abdomen. The injected flies were placed on the standard corn meal-agar-molasses medium.

Injection experimentation

Third instar larvae 72 hours ± 4 hours post-hatch were selected for injection. Samarkand and tu-h were used for these injections. The injection solutions used were:

1) Ringers + 0.02 mg/ml FU
2) Ringers + 5.0 mg/ml FU
3) Ringers + 15.0 mg/ml FU
4) Ringers + 0.02 mg FU + 0.20 mg TdR/ml
5) Ringers

Adults of both strains were also injected with:

1) Ringers + 5.0 mg/ml FU
2) Ringers + 15.0 mg/ml FU
3) Ringers + 30.0 mg/ml FU
4) Ringers

Experimentation to Determine the Mode of Transfer of Fluorouracil from Parents to Progeny

Larvae at 72 hours ± 4 hours post-hatch were placed on a sucrose-brewers yeast medium supplemented with 0.8 microcuries of FU-2-^{14}C* per gm of medium. Larvae of the same age were also placed on a non labeled medium. The larvae were allowed to complete their development to adults on the 2 media. Virgin adults were collected and the following crosses were made:

1) labeled males X non labeled females

2) non labeled males X labeled females

The first 25 eggs laid from each mating were collected and crushed on a planchet and analyzed for radioactivity using a Nuclear-Chicago Model D-47 Geiger Tube and a Nuclear-Chicago Model 8700 Counter.

Experimentation to Determine the Incorporation of Fluorouracil into RNA

Technique for labeling larvae

Large numbers of Samarkand larvae were collected and placed on the sucrose-brewers yeast medium defined earlier and supplemented with 0.8 microcuries of FU-2-^{14}C per gm of medium. The larvae were maintained on this medium for 8 hours then removed. To insure against contamination on the external body surfaces, the following washing procedure was implemented (Kilgore and Painter, 1962):

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*5-fluorouracil-2-^{14}C, SA = 20mc/mM, was obtained from Calbiochem

1) the larvae were washed twice in distilled water and centrifuged each time.
2) they were washed twice in 70% ethanol and centrifuged after each wash.
3) they were washed in a solution of 95% ethanol and ethyl ether (3:1 v/v) and centrifuged.
4) they were finally washed twice in ethyl ether.

Immediately after washing the larvae were frozen. To test the effectiveness of this washing procedure, non labeled larvae were placed on the labeled media and removed 5 minutes later. They were washed according to the above procedure and placed on a planchet. The whole larvae were analyzed for radioactivity using the Nuclear-Chicago counting system. Based on a t-test, there was no significant increase in radioactivity over background.

Extraction and hydrolysis of RNA

This extraction procedure was a slightly modified version of that given by Kilgore and Painter (1964).

Acid-soluble fraction. 200 mg of frozen labeled larvae were placed in a heavy walled 12 ml centrifuge tube and 0.1 ml of cold (5°C) 0.17N $HClO_4$ was added. The larvae were then homogenized for two minutes in an ice bath using a teflon pestle attached to an electric motor. Following the homogenization, 0.9 mls of cold 0.17N $HClO_4$ was added to wash down the sides of the tube and pestle and to dilute the sample. The diluted samples were then centrifuged at 7500 rpm for 20 minutes at 0°C. The supernatant was removed and saved. The residue was thoroughly mixed with 1.0 mls of cold $HClO_4$ and again centrifuged.

The two supernatant solutions, containing free FU and its acid-soluble metabolic products, were combined and saved for subsequent analysis. To make sure that all the FU which was not incorporated was washed out in this process, non labeled larvae were homogenized and 0.002 microcuries of FU-2-^{14}C was added to the homogenate. This homogenate was extracted twice with cold 0.17N $HClO_4$ and the acid-insoluble residue was analyzed for radioactivity. Based on a t-test, there was no significant increase in radioactivity over background.

Acid-insoluble fraction. The residue remaining after the cold $HClO_4$ extraction was extracted twice with 95% ethanol to remove lipids and waxy substances. After each extraction the samples were centrifuged and the supernatants kept for analysis. The nucleic acids were extracted by treating the residue with 2.0 mls of 0.5N $HClO_4$ for 20 minutes in a water bath at 90°C then centrifuging. The nucleic acids were removed as soluble products in the hot acid extract. The supernatant was kept for nucleic acid analysis.

Hydrolysis of the nucleic acid. The acid-soluble and the acid-insoluble fractions were made basic with 0.3N KOH (pH at 11) and incubated for 20 hours at 37°C. After the 20 hours, the samples were acidified with $HClO_4$ (pH at 6). A flow sheet for this extraction procedure is shown in Figure 3.

Paper chromatography

The solvent system used in all chromatographic work was composed of: n-butanol (3), 95% ethanol (2), and 5N HCl (2) (Kilgore and Painter,

1964). All samples were run on Whatman no. 1 paper for 24 hours. The chromatograms were cut up into pieces 19.05 mm and analyzed in the Nuclear-Chicago counting system. To determine areas where the radioactivity was concentrated, the chromatogram was run through a strip counter. This strip counter was manufactured by Garth Westenskow Co. of Salt Lake City.

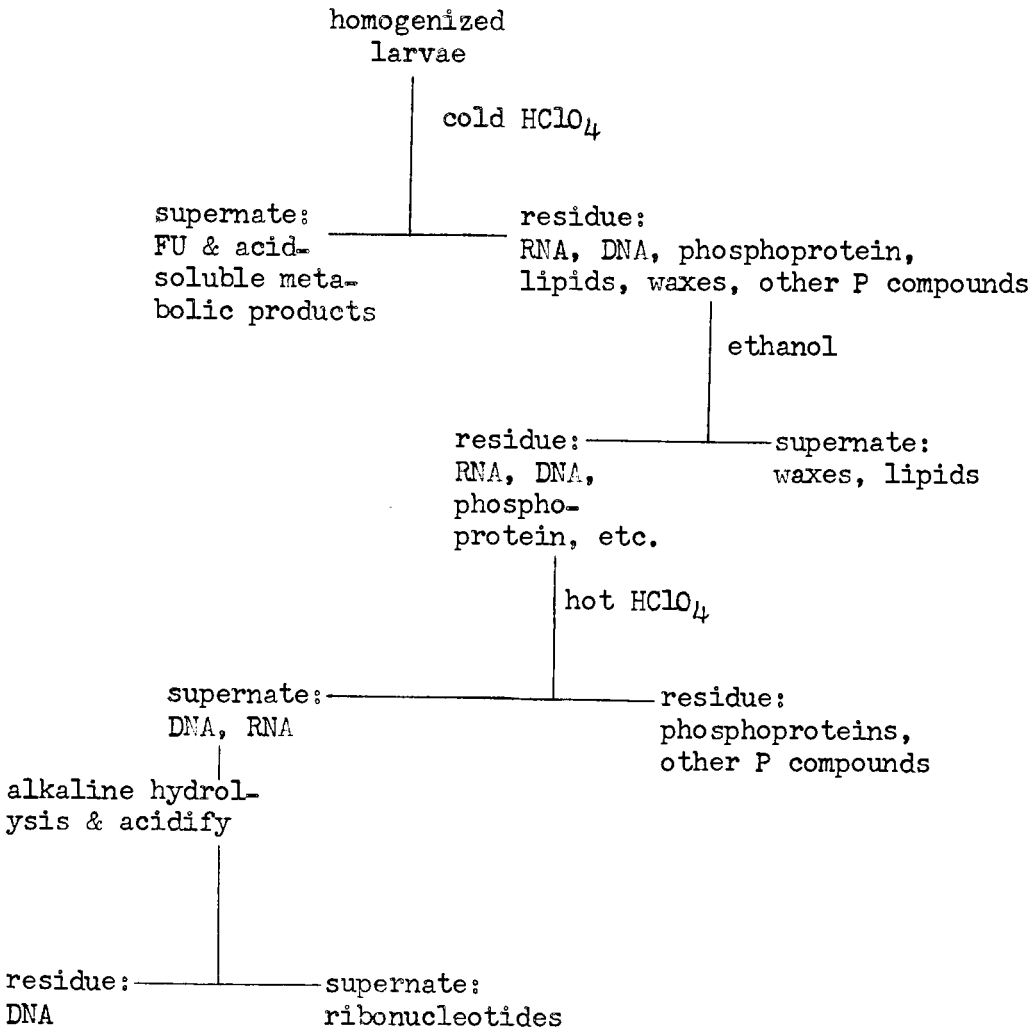

Figure 3. Flow sheet for the extraction of RNA

EXPERIMENTAL RESULTS

Effects of Fluorouracil on Eggs

When the eggs were held in a solution of Ringers + 0.02 mg/ml FU, the hatchability was significantly decreased (Table 1). Of the 337 eggs that were placed in the FU solution, only 137 hatched. It is also worthwhile to note that of the 137 eggs that hatched, 130 of them hatched between 24 and 48 hours post-lay. When the FU concentration was increased to 0.05 mg/ml, the hatchability was decreased even more. Of 474 FU treated eggs, only 94 hatched and 82 of these hatched between 24 and 48 hours post-lay. From 734 control eggs (those placed in Ringers) 340 hatched. A summary of the hatchability data has been constructed:

treatment	% hatch 0-24 hrs	24-48 hrs	total
Ringers + 0.02 mg/ml FU	2.1	38.6	40.7
Ringers + 0.05 mg/ml FU	2.5	17.3	19.8
Ringers	32.4	13.9	46.3

It was also noted that from the 0.02 mg/ml FU treated eggs, 63.5% of those that hatched finally pupated; from the 0.05 mg/ml FU treated eggs that percentage was considerably decreased to 14.9%. These values are in contrast to 77.9% pupation from the control eggs.

Once the larvae from the FU treated eggs had pupated, the effect of FU was no longer in evidence because the number of adults emerging from the pupae was not significantly different from the number of adults

that emerged from the pupae in the control experiment based on a chi-square test. Eighty-one adults emerged from the 101 pupae formed from the FU treated eggs and 243 adults emerged from the 265 pupae formed from Ringers treated eggs.

When the adults that emerged from the treated eggs were inbred for one generation (2 males X 2 females), the number of progeny produced was significantly lower in those parents that originated from the FU treated eggs (Table 2). The mean number of progeny produced by the parents from the FU treated eggs was 146.93, whereas the mean was 178.60 for the parents from the Ringers treated eggs.

The Effect of Fluorouracil when Ingested

Larvae fed the three different doses of FU (0.01 mg, 0.064 mg, and 0.12 mg per gm of media) were markedly inhibited in their development (Table 3). No mutations or phenocopies were noted in any of the adults that emerged. Samarkand seemed less sensitive to FU than tu-h, however, the type of reaction was the same in both strains. This reaction was characterized by a prolonged larval stage, and a toxic effect. Of the tu-h larvae that were placed on the media containing 0.01 mg FU per gm, only 13.7% reached the adult stage. This compares with 17.6% for Samarkand under identical conditions. When the FU concentration was increased to 0.64 mg per gm of media, only 0.18% of the tu-h and 0.82% of the Samarkand reached the adult stage. These values are in contrast to 79.2% and 93.5% for tu-h and Samarkand controls respectively. At an FU concentration of 0.12 mg per gm of medium, only small, unhealthy larvae, the size of early second instars, were recovered at 264 hours post-hatch.

The reaction of the larvae to the two different concentrations of TdR (2.4 mg and 12.0 mg per gm of medium) and to one concentration of UR (2.4 mg per gm of medium) was characterized by a slight toxicity to the lower concentration of UR and TdR and a much greater toxicity to the higher concentration of TdR.

When various combinations of TdR, UR, and FU were fed, there was no indication of any reversal of the inhibitory effect of FU up to 168 hours post-hatch, since at the termination of these experiments testing for reversal, only small, unhealthy larvae were recovered.

Injection Experimentation

The effect of injecting larvae and adults with FU was similar to the effect achieved when FU was fed, in that the compound was toxic to the organisms. Adults were much less sensitive to FU than larvae. A critical factor involved in the injection experiments was the survival of the organism following the injection. The rate of survival of the adults was essentially 100%, but the maximum survival rate for the larvae was 36.1% for Samarkand and 19.5% for tu-h (Table 4).

When a combination of FU and TdR was injected, no reversal of the toxic effect was noted.

Mode of Transfer of Fluorouracil from Parents to Progeny

When males, labeled with FU-2-^{14}C, were mated to non labeled females, the eggs produced by these females contained no significant levels of radioactivity. However, if non labeled males were mated to labeled

females, the eggs from this cross contained significant levels of radioactivity, based on a t-test (Table 5).

Analysis for Incorporation of Fluorouracil Into the RNA of Drosophila

The analysis of the RNA of the labeled larvae was subdivided into several aspects. R_f values were established for the five bases, adenine, guanine, cytosine, thymine, and uracil, as well as for their nucleosides and nucleotides in the solvent system used for chromatography (Table 6). An R_f value was also established for FU. Since a sample of FURP was not available, the R_f value of this nucleotide was generously furnished by Dr. Wendell W. Kilgore of the University of California, Davis, California.

The products of the extraction process were analyzed for radioactivity (Table 7), and it was found that the only fraction that did not contain a significant level of radioactivity was the ethanol extract, containing the lipids and waxy materials. Both the acid-soluble and the acid-insoluble fractions contained significant levels of radioactivity as did the acid-insoluble-non-lipid-phosphorus (AINLP) residue remaining after the hot $HClO_4$ extraction.

The RNA analysis involved the chromatography of the AHAI and AHAS fractions. The chromatogram of the AHAI fraction showed 5 UV absorbing spots (carrier FU being one of them), while the chromatogram of the AHAS fraction showed only one UV absorbing spot corresponding to the carrier FU. The R_f values calculated for each spot were:

	AHAI fraction	AHAS fraction
1)	.771	.759
2)	.685	
3)	.496	
4)	.313	
5)	.213	

These values compare with the R_f values of the deoxyribonucleotides and FU in Table 6. The R_f values for the ribonucleotides were not available but by analogy with the R_f values for the deoxyribonucleotides it seemed as if the 4 nucleotides of RNA were present in the AHAI fraction.

The chromatogram of each fraction was cut up into 19.05 mm pieces, numbered accordingly from the bottom to the top, and analyzed for radioactivity(Tables 8 and 9). Significant levels of radioactivity were found in areas 11 and 12 of the chromatogram of the AHAI fraction and in areas 9, 11, 12 and 13 of the AHAS fraction. The carrier FU spot was located in areas 10 and 11 of each chromatogram, therefore area 12 of the AHAI fraction and areas 9, 12, and 13 of the AHAS fraction contained radioactivity not associated with the FU spot.

In order to more precisely localize the areas of radioactivity on the chromatograms, the strip counter was utilized. The results of the strip counter analysis can be seen in Figures 4 and 5. The figures have been partitioned and numbered according to the areas analyzed in the Nuclear-Chicago counting system. The numbers in the figures refer to the numbers in Tables 10 and 11. Note that there was one peak of activity from the AHAI fraction and two broad peaks from the AHAS fraction. When the chromatograms were cut up, it was found that the

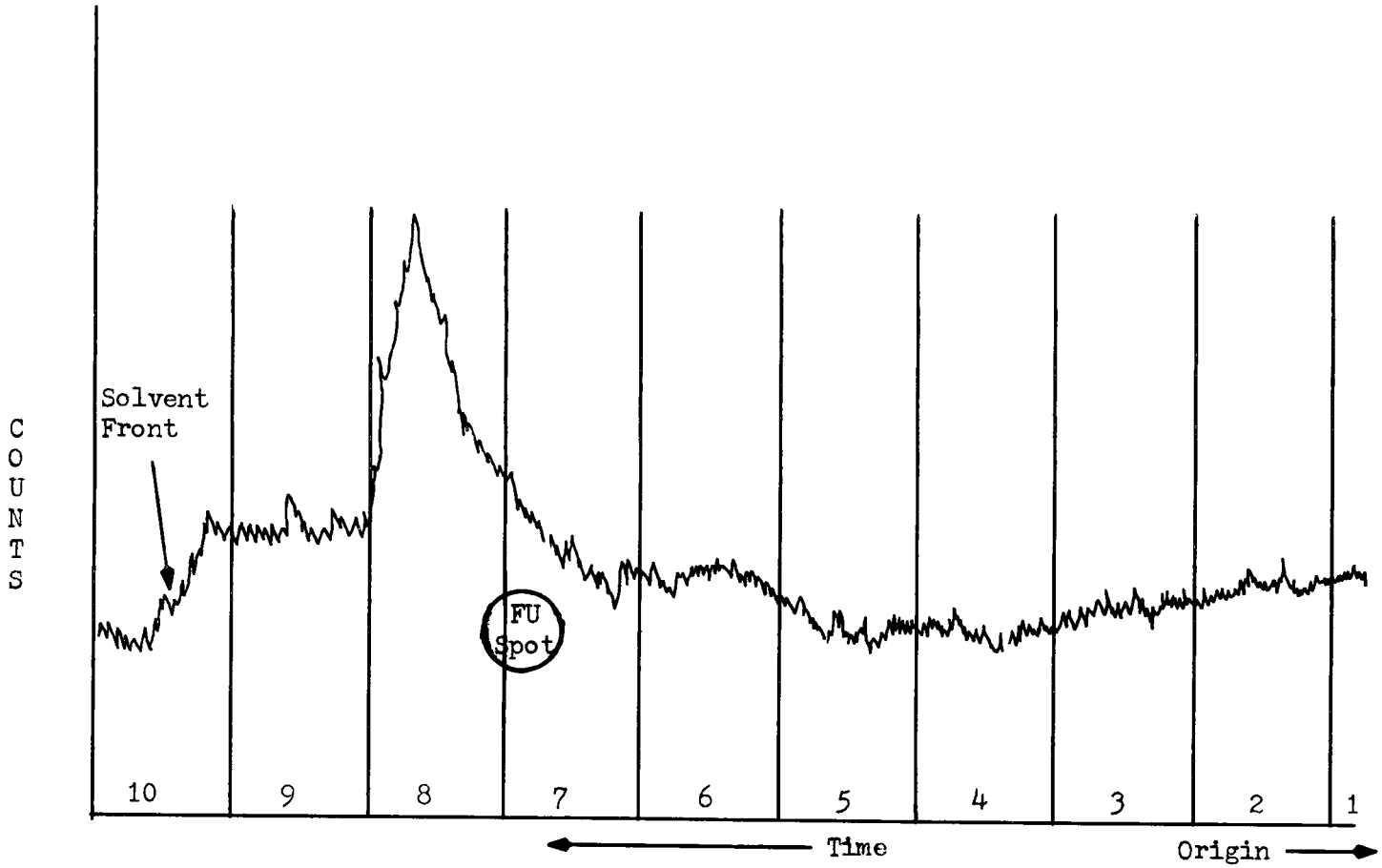

Figure 4. Results of the analysis of the AHAI fraction chromatogram with a strip counter. The partitions refer to the areas analyzed in the Nuclear-Chicago counting system (Table 11)

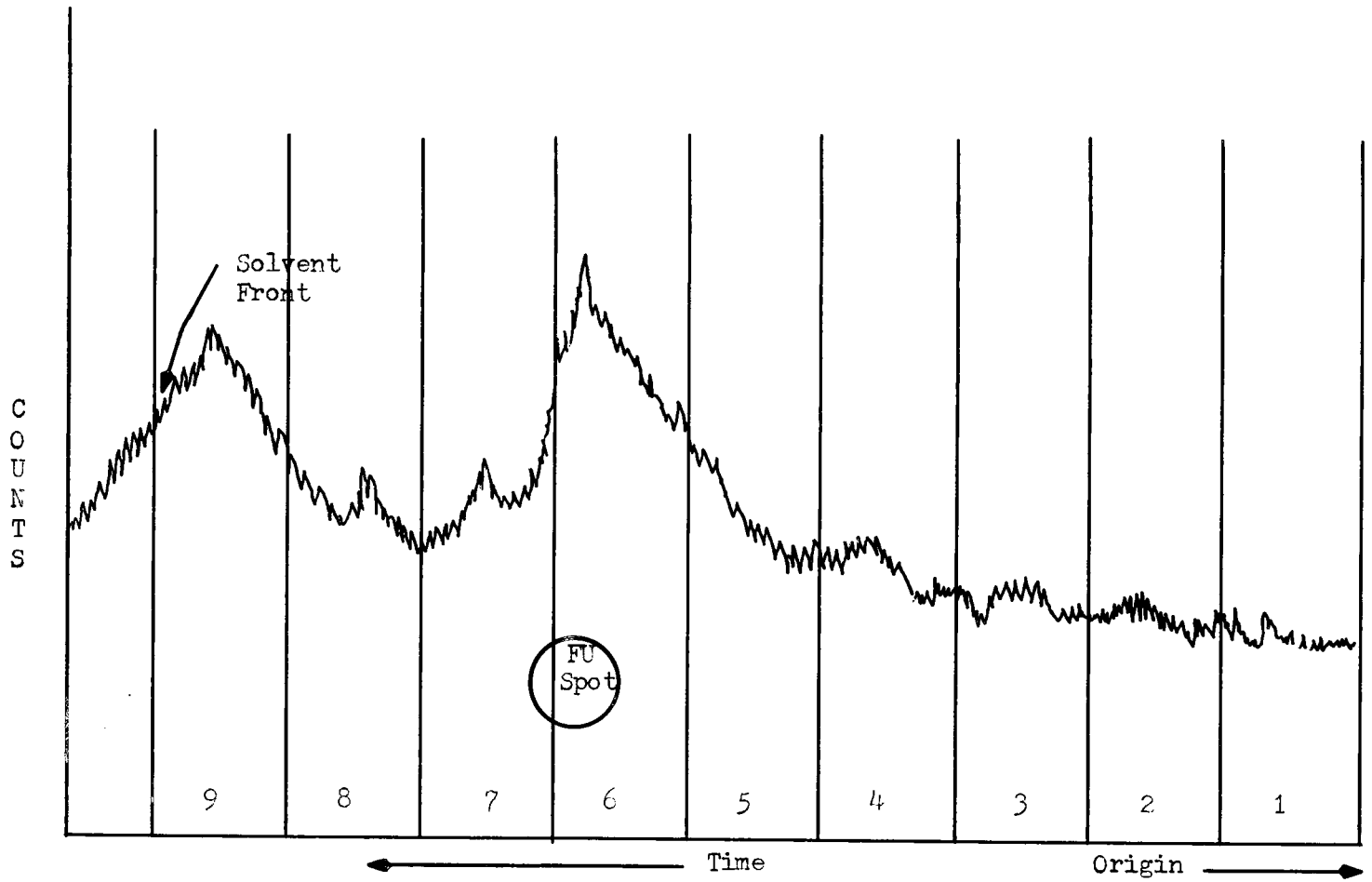

Figure 5. Results of the analysis of the AHAS fraction chromatogram with a strip counter. The partitions refer to the areas analyzed in the Nuclear-Chicago counting system (Table 10)

areas corresponding to the peaks of radioactivity indeed showed significant levels of radioactivity. Incidentally, the R_f values for the carrier FU did not compare well with the value recorded in Table 6, but this is probably due to variations in the properties of the chromatographic system.

In order to verify the fact that the radioactivity in the chromatogram was not coming from the FU but rather from the area just above it where FURP would be, a chromatogram was run of the AHAI fraction with carrier FU. The chromatogram was carefully analyzed with UV light in order to determine the total area occupied by the FU spot. This spot was then carefully cut out and analyzed for radioactivity. The amount of radioacitivity in this spot was significantly above background (Table 12). The area immediately above the FU spot was also cut out and analyzed and it too had a level of radioactivity significantly above background. Next the centers of the FU spot and the area just above it were cut out and analyzed and it was found that the center of the FU spot was not radioactive but the center of the area above it was. If FU was the source of the radioactivity, then that radioactivity should be concentrated in the center of the FU spot. This was not the case. If, on the other hand, FURP was the source of the radioactivity, then that radioactivity would be concentrated in the center of the area immediately above the FU spot. This was indeed the case.

DISCUSSION

The eggs that were soaked in a Ringers + FU solution were inhibited in their development. The higher the concentration of FU, the greater was the degree of inhibition. The larvae that were fed the compound also manifested a marked degree of inhibition, an inhibition that was more pronounced when the concentration of FU was increased. The most obvious effect of FU when it was fed to the larvae was a greatly extended larval period. As a matter of fact, a great many larvae on a medium containing 0.12 mg FU per gm were the size of early second instars at 264 hours post-hatch. Of course, concurrent with the protracted larval period was a toxic effect. The results of the injection experiments also demonstrated the same type of toxic effect. The conclusion would be that FU, whether ingested with the medium or injected into the body cavity, produces an inhibitory and toxic effect. The adult Drosophila were not nearly as sensitive to FU as were the larvae and this was, perhaps, understandable since the larvae were metabolically more active than adults. A sideline of the larval injection and feeding experiments was the fact that no visible mutations or phenocopies were observed in the adults.

All attempts to reverse the inhibitory effect of FU proved to be futile. Exogenous sources of TdR and UR and combinations of the two had no positive effect in eliminating the FU toxicity. TdR was toxic by itself at a concentration of 12.0 mg per gm of media. At concentrations of 2.4 mg per gm of media, TdR and UR were slightly toxic.

These effects agree with the results obtained by Allen (1962), when testing the effects of DNA base analogs on Drosophila. If FU were metabolized to FUdRP by Drosophila, if thymidylate synthetase were inhibited by that deoxyribonucleotide, and if this were the primary mode of action of FU in Drosophila, then the exogenous TdR should bypass the metabolic block and allow the organism to develop normally. The fact that this was not the case indicates that the inhibitory effect of FU can't be attributed primarily to the blockage of the methylating enzyme. Blockage of this enzyme by FUdRP, however, can't be ruled out entirely as a contributing cause of inhibition because the inhibitory effect may be due to a combination of factors, such as blockage of the enzyme and a toxic response to the products of FU catabolism. Assuming that this is the case, then relieving the enzyme inhibition with TdR would still leave the products of FU catabolism to exert their toxic effect. However, since in all attempts to relieve FU toxicity with TdR there was no hint of reversal, one could conclude that if there exists a blockage of the methylating enzyme, then it must exert a minor influence in the overall inhibitory effect.

The investigation of the RNA of the labeled larvae proved to be quite fruitful. Analysis of the chromatograms of the AHAI and AHAS fractions revealed areas of significant radioactivity. The radioactivity in the AHAS fraction was spread out over a wide area and this was to be expected since in this fraction would be found the free FU and some of its acid-soluble metabolic products. The radioactivity in the AHAI fraction was concentrated in the area just ahead of the carrier FU spot. This, too, was to be expected if FURP was present,

but since FU and FURP would be very close to each other on the chromatogram, the possibility of some labeled FU overlapping with FURP had to be eliminated. This was done by removing the center of the FU spot and analyzing it for radioactivity, and then removing the center of the area just above the FU spot and similarly analyzing it. The results showed the radioactivity to be concentrated in the area just above the FU spot, whereas the center of the FU spot showed no significant level of radioactivity. The range of R_f values for the area above the FU spot was .722 - .845. The R_f value for FURP obtained from Kilgore fits into this range. The conclusion that can be drawn from the RNA analysis is that FU is incorporated into the RNA of Drosophila. Unfortunately, based on the data presented, it would be presumptuous to conclude that the incorporation of FU into RNA was the cause of the inhibition, but there is a strong possibility that it was, since alteration of the sequence of bases in the RNA molecules would likely cause an altered protein to be formed. Investigations into protein synthesis in Drosophila that have FU incorporated into their RNA will be necessary before positive statements concerning the effect of FU substitution in Drosophila RNA can be made.

The data from the experiments that determined the mode of transfer of FU from parents to progeny supports the contention that FU is incorporated into RNA and not into DNA because if DNA were FU-substituted, the eggs from the cross between labeled males and nor labeled females would have been labeled.

SUMMARY

1) Investigations with FU and its effects on Drosophila melanogaster showed that the compound had a marked inhibitory effect on development and was toxic.

2) Experiments designed to elucidate the mode of action of FU in Drosophila were performed and the results indicated that the fluorinated pyrimidine is incorporated into the RNA. All evidence indicated that thymidylate synthetase was not inhibited to any great extent by FU.

3) Incorporation of FU into RNA does not prove that the toxic effect of the compound is due to the production of a non functional RNA but it is indicative.

4) FU is passed from parents to progeny through the egg and not the sperm.

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APPENDIX

Table 1. The effect of 5-fluorouracil on the eggs of tu-h

Treatment	No. of eggs	No. that hatch		Pupate	Adults
		0-24 hrs	24-48 hrs		
Ringers + 0.02 mg/ml FU	337	7**	130*	87**	71^{ns}
Ringers + 0.05 mg/ml FU	474	12**	82**	14**	10^{ns}
Ringers	734	238	102	265	243

*significant to the 0.05 level based on a chi-square test

**significant to the 0.01 level based on a chi-square test

^{ns} not significant based on a chi square test

Table 2. A comparison of the number of progeny produced by parents from 0.02 mg/ml FU treated eggs with the progeny produced by parents from Ringers treated eggs. (2 male and 2 female parents)

Parents	Set no.	No. progeny
from eggs treated in Ringers	1	182
	2	162
	3	201
	4	171
	5	171
	6	194
	7	179
	8	178
	9	193
	10	159
	11	176
	12	181
	13	168
	14	177
ave. number of progeny produced per set = 178.00		
from eggs treated in Ringers + 0.02 mg/ml FU	1	147
	2	152
	3	143
	4	151
	5	135
	6	152
	7	146
	8	138
	9	170
	10	160
	11	132
	12	139
	13	147
	14	150
ave. number of progeny produced per set = 147.18		
Based on a t-test ($t = 7.373$), the difference is highly significant		

Table 3. Percentage of larvae that pupated based on a minimum sample of 200 larvae. Observations were made at the 24 hour intervals indicated

Supplements per gm of medium and strain used		Hours post-hatch 96	120	144	168	192	216	240	264
0.01 mg FU	tu-h	1.17	16.1	0	0	0	0	9.8a	3.97a
	Sam	1.95	18.0	0	0	0	0	12.8a	4.76a
0.064 mg FU	tu-h	0	0	0	0	0	.18	0	.18a
	Sam	0	0	0	0	0	.91	0	.82a
0.12 mg FU	tu-h	0	0	0	0	0	0	0	0
	Sam	0 0	0	0	0	0	0	0	0
2.4 mg TdR	tu-h	1.0	43.5	18.5	0	X	X	X	X
	Sam	16.5	63.5	0	0	X	X	X	X
12.0 mg TdR	tu-h	0	0	0	2.6	X	X	X	X
	Sam	0	0	0	8.3	X	X	X	X
2.4 mg UR	tu-h	6.5	58.0	0	0	X	X	X	X
	Sam	25.5	61.5	0	0	X	X	X	X
0.12 mg FU + 2.4 mg TdR	tu-h	0	0	0	0	X	X	X	X
	Sam	0	0	0	0	X	X	X	X
0.12 mg FU + 12.0 mg TdR	tu-h	0	0	0	0	X	X	X	X
	Sam	0	0	0	0	X	X	X	X
0.12 mg FU + 2.4 mg UR	tu-h	0	0	0	0	X	X	X	X
	Sam	0	0	0	0	X	X	X	X

continued on next page

Table 3. continued

Supplements per gm of medium and strain used		Hours post-hatch 96	120	144	168	192	216	240	264
0.12 mg FU + 2.4 mg UR + 2.4 mg TdR	tu-h	0	0	0	0	X	X	X	X
	Sam	0	0	0	0	X	X	X	X
control (no additive)	tu-h	27.3	56.4	0	0	75.0a	4.4a	X	X
	Sam	34.2	61.4	0	0	87.6a	5.2a	X	X

a = adults that emerged
X = experiment was terminated at the hour preceding the X

Table 4. Injection of larvae and adults with FU, FU + TdR, and Ringers

Developmental stage & strain	Injection solution per ml[a]	No. injected	No. survivors 5 hrs	10 hrs	No. pupate
tu-h larvae at 72 hrs post-hatch	0.02 mg FU	201	8	3	1
	5.0 mg FU	71	9	0	0
	15.0 mg FU	42	2	0	0
	0.02 mg FU + 0.20 mg TdR	97	5	0	0
	control (no additive)	307	60	55	53
Sam larvae at 72 hours post-hatch	0.02 mg FU	160	41	30	16
	5.0 mg FU	63	12	2	0
	15.0 mg FU	41	3	0	0
	0.02 mg FU + 0.10 mg TdR	103	21	15	6
	control (no additive)	291	105	99	95

Developmental stage & strain	Injection solution per ml[a]	No. injected	No. survivors 12 hrs	10 hrs	No. pupate
tu-h adults	5 mg FU	81	81	66	--
	15 mg FU	80	80	55	--
	30 mg FU	50	36	30	--
	control (no additive)	47	47	47	--
Sam adults	5 mg FU	75	75	64	--
	15 mg FU	71	71	53	--
	30 mg FU	62	47	31	--
	control (no additive)	51	51	51	--

[a] basic injection solution was insect Ringers

Table 5. Analysis for radioactivity of the eggs from crosses involving: 1) labeled ♂ x non-labeled ♀ and 2) non-labeled ♂ x labeled ♀

1) Labeled ♂ x non-labeled ♀

Planchet without sample: cpm	Planchet with sample: cpm	S	T	Signif.
17.70	19.17	1.47	1.30	ns

2) Non-labeled ♂ x labeled ♀

Planchet without sample: cpm	Planchet with sample: cpm	S	T	Signif.
18.20	24.38	1.06	8.25	s (.01)

Table 6. R_f values using the n-butanol, ethanol, HCl solvent system

Compound	R_f
Adenine	.314
Adenosine	.316
Adenosine Monophosphate	.318
Guanine	.202
Guanosine	.201
Guanosine Monophosphate	.203
Cytosine	.396
Cytidine	.471
Cytidine Monophosphate	.511
Thymine	.828
Thymidine	.853
Thymidine Monophosphate	.882
Uracil	.669
Uridine	.572
Uridine Monophosphate	.688
5-Fluorouracil	.759
5-Fluorouridine Monophosphate	.810

Table 7. Analysis for radioactivity of the extracts from the RNA extraction procedure

a) Planchet without sample

No.	Cpm
1	16.85
2	16.75
3	17.50
4	18.02

b) Planchet with sample

No. and description	Cpm	pm per ml or gm	S	T	Sig.
1) 10λ alcohol extract	18.90	205	1.38	2.09	ns
2) 1 mg AINLPa	21.63	488	1.24	5.61	s(.01)
3) 10λ acid-insoluble fraction	24.30	680	.99	9.62	s(.01)
4) 10λ acid-soluble fraction	30.06	1204	1.72	9.90	s(.01)

[a]AINLP = acid-insoluble-non-lipid-phosphorus

Table 8. Analysis for radioactivity of the chromatogram of the AHAI fraction. The chromatogram was cut up into pieces at 19.05 mm intervals beginning at 6.35mm below the origin of the spot

Planchet without sample:

No.	Cpm
1	18.60
2	18.00
3	16.70
4	17.97
5	17.80
6	19.03
7	19.33
8	17.53
9	16.33
10	17.90
11	18.03
12	17.40
13	16.80
14	18.63
15	17.43

Planchets with sample:

No. and range in mm covered by the sample	Cpm	S	T	Signif.
1) -6.35 - 12.70	17.80	1.43	.61	ns
2) 12.70 - 31.75	18.30	3.00	.12	ns
3) 31.75 - 50.80	17.23	1.90	.34	ns
4) 50.80 - 69.85	18.27	.68	.55	ns
5) 69.85 - 88.90	16.63	.75	1.95	ns
6) 88.90 - 107.95	17.90	1.26	1.07	ns
7) 107.95 - 127.00	17.17	1.98	1.34	ns
8) 127.00 - 146.05	17.80	1.51	.22	ns
9) 146.05 - 165.10	18.33	1.50	1.63	ns
10) 165.10 - 184.15[a]	19.07	1.94	.74	ns
11) 184.15 - 203.20[a]	21.67	1.32	3.37	s(.05)
12) 203.20 - 222.25	25.10	2.50	3.76	s(.01)
13) 222.25 - 241.30	18.27	1.06	1.71	ns
14) 241.30 - 260.35	18.80	1.92	.11	ns
15) 260.35 - 279.40	17.70	1.27	1.23	ns

[a]FU spot was found in these areas

Table 9. Analysis for radioactivity of the chromatogram of the AHAS fraction. The chromatogram was cut up into pieces at 19.05 mm intervals beginning at 6.35mm below the origin of the spot

Planchets without sample: No.	Cpm
1	18.77
2	17.80
3	17.20
4	17.60
5	17.00
6	18.40
7	19.07
8	17.50
9	18.10
10	17.00
11	15.90
12	18.93
13	18.17
14	17.07
15	18.00

Planchets with samples: No. and range in mm covered by the sample	Cpm	S	T	Signif.
1) -6.35 - 12.70	17.87	1.57	.70	ns
2) 12.70 - 31.75	18.13	1.62	.25	ns
3) 31.75 - 50.80	18.03	1.33	.76	ns
4) 50.80 - 69.85	18.18	1.70	.45	ns
5) 69.85 - 88.90	17.03	1.06	.04	ns
6) 88.90 - 107.95	19.53	1.30	1.14	ns
7) 107.95 - 127.00	17.33	1.52	1.50	ns
8) 127.00 - 146.05	18.27	1.42	1.18	ns
9) 146.05 - 165.10	22.28	1.79	3.05	s(.05)
10) 165.10 - 184.15	18.80	1.98	1.19	ns
11) 184.15 - 203.20	21.70	1.51	5.00	s(.01)
12) 203.20 - 222.25	21.38	.90	3.55	s(.02)
13) 222.25 - 241.30	21.95	1.37	3.60	s(.02)
14) 241.30 - 260.35	19.05	1.80	1.45	ns
15) 260.35 - 279.40	17.20	1.46	.67	ns

Table 10. Analysis of the chromatogram of the AHAS fraction for radioactivity after it was analyzed in a strip counter. Refer to Figure 5 for the areas covered by the numbers

Planchets without samples:

No.	Cpm
1	17.03
2	16.93
3	17.20
4	17.93
5	18.13
6	18.60
7	18.30
8	16.63
9	17.70

Planchets with samples:

No.	Cpm	S	T	Signif.
1	18.13	1.55	0.99	ns
2	18.26	1.38	1.36	ns
3	18.45	2.38	0.74	ns
4	18.57	1.34	0.68	ns
5	18.47	0.71	0.68	ns
6	23.98	1.13	6.64	s(.01)
7	19.92	1.58	1.45	ns
8	24.30	1.55	6.90	s(.01)
9	23.88	1.94	4.51	s(.01)

Table 11. Analysis of the chromatogram of the AHAI fraction for radioactivity after it was analyzed in the strip counter. Refer to Figure 4 for the areas covered by the numbers

Planchets without samples:

No.	Cpm
1	18.87
2	18.40
3	17.93
4	17.83
5	18.93
6	18.73
7	18.83
8	17.60
9	17.03
10	17.93

Planchets with samples:

No.	Cpm	S	T	Signif.
1	17.72	1.05	1.55	ns
2	18.60	1.63	0.17	ns
3	18.68	2.01	0.52	ns
4	17.30	1.20	0.62	ns
5	17.88	1.46	0.99	ns
6	18.34	1.21	0.38	ns
7	20.04	1.29	1.27	ns
8	35.94	1.62	15.68	s(.01)
9	18.76	2.09	1.14	ns
10	17.94	0.91	0.01	ns

Table 12. Detailed radioactivity analysis of the FU spot and the area immediately above it on the chromatogram of the AHAI fraction

Planchets without samples:

No.	Cpm
1	17.03
2	16.93
3	17.20
4	17.93

Planchets with samples:

No. and description	Cpm	S	T	Signif.
1) entire FU spot	25.40	1.33	8.6	s(.01)
2) entire area above FU spot	29.34	1.97	8.63	s(.01)
3) center of FU spot	18.80	1.98	1.10	ns
4) center of area above FU spot	23.38	1.49	4.78	s(.01)