J. of Korean Ind. & Eng. Chemistry,
Vol. 2, No. 1, March 1991, 56－63.

Physiological Genetic Effects of Monomer and Polymer Containing 5-Fluorouracil on Drosophila Melanogaster

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(*Received February 25, 1991*)

5-플루오로우라실을 포함하는 단량체와 중합체의 노랑초파리에 대한 생리유전학적 영향

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(1991 년 2 월 25 일 접수)

Abstract : The monomer, 1-(2-carbomethoxyacryloyl)-5-fluorouracil(CMAFU) was synthesized from trans-β-carbomethoxyacryl chloride and 2, 4-bis(trimethylsilyloxy)-5-fluoropyrimidine. A copolymer of CMAFU with methyl methacrylate (MMA) [poly(CMAFU-co-MMA)] was also prepared with 2, 2′-azobisisobutyronitrile in cyclohexanone at 60 °C. Physiological genetic effects of 5-fluorouracil(5-FU), CMAFU and poly(CMAFU-co-MMA) on *Drosophila melanogaster* were investigated by the adult feeding method of Lewis and Bacher. It was found that a physiological genetic effect on the *Drosophila melanogaster* was considerably weaker for CMAFU and its copolymer than 5-FU.

요 약

1-(2-carbomethoxyacryloyl)-5-fluorouracil(CMAFU) 을 trans-β-carbomethoxyacryl chloride 와 2, 4-bis(trimethylsilyloxy)-5-fluoropyrimidine 으로부터 합성하고, CMAFU 와 메틸메타크릴레이트를 cyclohexanone 을 용매로 사용하여 60 °C 에서 라디칼공중합을 하였다. 5-FU, CMAFU 및 poly(CMAFU-Co-MMA) 들이 노랑초파리에 미치는 생리유전학적 영향을 Lewis 와 Bacher 방법에 의하여 조사한 결과, CMAFU 와 그 중합체가 5-FU 보다 생리유전학적 영향이 약함을 알 수 있었다.

1. INTRODUCTION

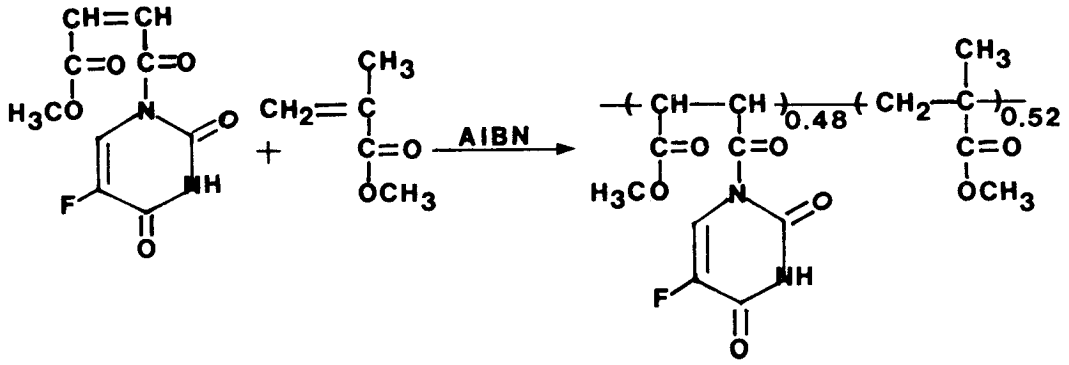
The mutagenic activity of some chemical mutagens in *Drosophila* has been studied by several investigators after Auerbach and Robson[1] reported the production of mutation by sulfur mustard. The alkylating agents such as methyl methanesulfonate(MMS), dimethylsulfonate(DMS) and ethyl methanesulfonate (EMS) were remarkably mutagens known to produce base-pair substitutions and chromosome changes. Especially EMS is known to show potential hazard when fed to adult *Drosophila* males as first shown by Alderson[2].

The inhibitory properties of 5-fluorouracil(5-FU) for tumor growth and its use in cancer chemotherap have resulted in extensive work on related compounds. It was observed, however, that 5-FU has strong side effect such as gastrointestinal toxicity and delivery problems[3, 4]. Lots of investigations on the metabolism and biochemistry of 5-FU have been reported, but the mechasnism by which it exerts its primary cytotoxic effects has not yet been clearly established [3~7]. Duke and Glassman[8] found streptomycin sensitive strain and fluorouracil resistant strain with drug effects in *Drosophila*.

Recently many attempts have been made to reduce toxic effects of 5-FU by introducing it as part of a polymer backbone or as a pendant group on a polymer chain. Umrigar et al.[9] synthesized 1-(2-carbomethoxyacryloyl)-5-fluorouracil(CMAFU) and the copolymers of CMAFU with vinyl monomers such as styrene, 2-chloroethyl vinyl ether and vinyl ether. Gebelein and Morgan[10] synthesized 5-fluoro-N-(N-allylcarbamoyl) uracil, 5-fluoro-N-(N-vinylcarbamoyl) uracil, and their polymers. Akashi et al.[11, 12] reported on the syntheses of N-methacryloyl oxyethyl-5-fluorouracil, 1-N-acryloyl-5-fluorouracil, 1-N-methacryloyl-5-fluorouracil, and their polymers and copolymers. They also studied on the in vivo antitumor activity of the polymers in Ehrlich's ascites tumor cells and found that the antitumor activity of polymers is greater than an equivalent amount of 5-FU alone.

Even though many researches have been reported on the biological activity of polymeric drugs, few intensive works to test the toxicity of the polymeric drugs themselves have been reported in an open literature. Furthermore no work to reveal the physiological genetic effect of polymeric drugs containing 5-FU in *Drosophila melanogaster* have been published.

The present study undertaken to clarify the effects of CMAFU and its copolymer treated in *D. melanogaster*. Physiological genetic effects in *D. melanogaster* were investigated in terms of mortality, egg to adult viability, fecundity, sex ratio, and developmental time by the adult feeding method of Lewis and Bacher[13]. For this work, we synthesized CMAFU and prepared a copolymer of CMAFU with methyl methacrylate by radical polymerization at 60 °C.



Scheme 1

2. EXPERIMENTALS

2. 1. 1-(2-Carbomethoxyacryloyl)-5-fluorouracil(CMAFU)

CMAFU was prepared by reacting 2, 4-bis(trimethylsilyloxy)-5-fluoropyrimidine with trans-β-carbomethoxyacryloyl chloride(TCMAC) in acetonitrile, as described by Umrigar et al.[9](m.p. : 148−149 °C, lit. m.p. : 150−151 °C).

Analysis : Calc. for $C_9H_7N_2O_5F$(242.2) : C, 44.63 ; H, 2.91 ; N, 11.57 %. Found : C, 44.47 ; H, 2.71 ; N, 11.60 %. IR(KBr, cm^{-1}) : 3450(−NH), 3080(=CH), 2840(aliphatic C−H), 1725 & 1705(−C=O), 1250(−C−O), and 815(NH). ^{1}H-NMR(acetone-d_6) : δ 8.2(d, 6H of pyrimidine ring), 7.8 & 6.7(d, 2H of ethylenic hydrogen) and 3.7ppm(s, 3H, $-CH_3$)

2. 2. Poly(1-(2-carbomethoxyacryloyl)-5-fluorouracil-co-methylmethacrylate) [Poly(CMAFU-co-MMA)]

Copolymerization of CMAFU with methyl methacrylate was carried out with AIBN in cyclohexanone at 60 °C. A solution of 2.42g(0.01 mol) CMAFU, 1.00 g (0.01 mol) MMA and 0.205 g AIBN in 25 ml dry cyclohexanone was introduced into a dry polymerization tube. The tube was sealed after degassed twice by purging with purified N_2 gas and placed in a regulated thermostat at 60 °C for specified periods. The polymer obtained was precipitated in excess n-hexane. The precipitate was collected by filtration and dried until a constant weight under vacuum. Analysis : Calc. for $C_{14}H_{15}N_2O_7F$: C, 49.10 ; H, 4.41 ; N, 8.24 %. Found : C, 51.39 ; H, 4.64 ; N, 8.02 %.

For biological test, poly(CMAFU-co-MMA) containing 48 mole % of CMAFU was used. Yield of the copolymer was 12 %. The intrinsic viscosity of the polymer was found to be 0.09, which was measured in N, N'-dimethylformamide at 30 ± 0.01 °C with Cannon-Fenske viscometer.

2. 3. Physiological genetic effects

The wild stock of *D. melanogaster* Oregon R(OR) males and its mutant *yumf & yf* : =were used for physiological effect tests. Throughout the experiment, *D. melanogaster* was cultured in the standard corn meal medium mixed with agar, yeast, and brown sugar in a 3×10 cm vial. Except when needed for counting or transferring, the cultures were kept in a constant-temperature cabinet at 25 °C over all experimental runs.

2. 3. 1. Mortality test

Experiments were carried out in 5 groups. For control groups, OR adult males were fed with only sterile 5 % sucrose solution in ethanol-water(1 : 1, v/v) mixture. For other experimental groups, 300 ppm or 1000 ppm of testing materials was added to the treatment solution, respectively. For convenience, the test solution consisting of 5-FU is designated as experimental group I, whereas other solutions containing CMAFU or poly(CMAFU-co-MMA) are designated as experimental groups II and III, respectively. Every 12 hr the number of dead files is scored for each experimental group. The treatment lasts for 504 hrs.

2. 3. 2. Physiological Effect

The effects of the test solutions on the viability, fecundity, and developmental time have been examined. The chemicals treatment followed essentially the method of Lewis and Bacher[13].

In control group, five treated males, mated with five OR virgins, were put into a fresh culture medium and eight simultaneous replications were made. At 24 hrs after the cross, all parental flies were tranferred to a second vial. Therefore twelve successive vials were obtained, and at 48 hr intervals, old females were discarded and five new virgins were supplied. The number of egg laid on the medium was counted every day immediately after the parental flies were transferred to the next vial. The count was made using mixed medium with green edible colouring for convenience. Number of adults from each vial was counted until the 16th day after the egg laid. From the relation of adult to egg number, viability, develop-

mental time and fecundity were counted.

In other experimental groups, the experimental procedure was done in the same manner as in the control group, except that the flies were fed with solution. Each testing solution was taken up with a pipette inserted in the suction pump and injected into tissue paper at the bottom of the vial. Sixty adult OR males were left in this vial for 24 hr. After 24 hr, treated OR males were crossed with virgin OR in five mating pairs. 10 replications were prepared.

2. 3. 3. Sex-linked recessive mutation

The effect of synthesized materials on the X-chromosome of *D. melanogaster* was measured by the sex ratio in F_1 after crossing *yf* ♀ =females with OR males. OR flies were reared for one generation with a medium containing 33 ppm of each experimental group. Five treated males were crossed with five virgin attached X females in a vial with the standard medium. They were transferred every 2 days until 4 successive vials were obtained. The numbers of flies who have emerged was counted every other day for 12 days.

3. RESULTS AND DISCUSSION

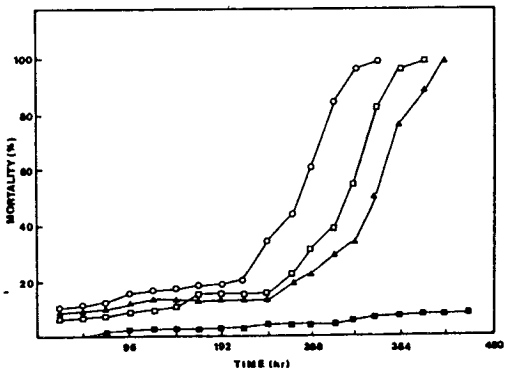


Fig. 1. Exposure-mortality relationship to various chemical compounds of OR males treated with various chemicals after adult feeding (1000 ppm).
■ : control group ; ○ : 5-FU ;
▲ : CMAFU ; □ : poly(CMAFU-co-MMA)

3. 1. Physiological Genetic Effect : Mortality

The effect of 5-FU, CMAFU and poly(CMAFU-co-MMA) on the mortality of *Drosophila melanogaster* is shown in Fig. 1. For Control group there is no significant change in mortality up to 480 hrs. However, the mortality of *D. melanogaster* treated with other experimental group increased remarkably after about 250 hrs. The lethal time at which all treated males were died is 360 hrs, 432 hrs and 408 hrs for 1000 ppm of experimental group I(5-FU), group II (CMAFU) and group III(CMAFU-co-MMA), respectively. We found, from this experiment, that the toxicity decreased in the order 5-FU > poly(CMAFU-co-MMA) > CMAFU > control.

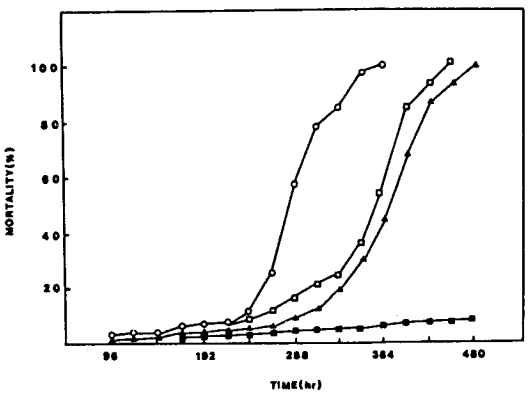


Fig. 2 Exposure-mortality relationship to various chemical compounds of OR males treated with various chemicals after adult feeding(300 ppm).
■ : control group ; ○ : 5-FU ;
▲ : CMAFU ; □ : poly(CMAFU-co-MMA)

It was also shown in Fig. 2 that the toxicity of 300 ppm of chemicals in *D. melanogaster* followed the same order as that of 1000 ppm of chemicals. Careful examinations of the two figures manifests that the toxicity of chemicals is slightly high as their concentration in the treatment medium is high. It should be noted that the mortality of the flies treated with 5-FU is significantly higher than those treated with any other experimental groups even at low concentra-

tion(300 ppm).

3. 2. Physiological Effect

3. 2. 1. Egg to adult viability

One-day-old adult males were treated with 33 ppm of each experimental group dissolved in 5 % sucrose solution for 24 hrs. Then, the parental flies were transferred to the next vial. Immediately, the number of eggs laid on the food medium was counted with a stereoscopic microscope(Kyowa model STZ-P). On the 9−10 days after the eggs were laid, adult flies began to emerge. They were daily counted after emergence. The emerged flies and laid egg number obtained from here was measured in terms of egg to adult viability. Fig. 3 shows the egg to adult viability of *D. melanogaster* treated with control or each experimental group.

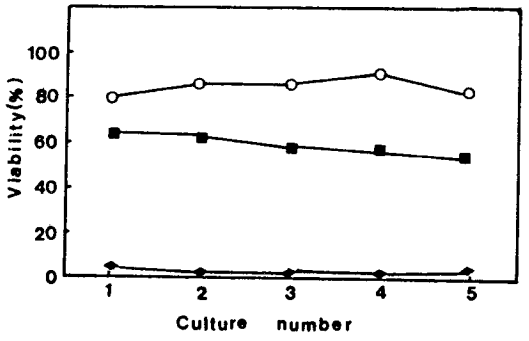


Fig. 3 Egg to adult viability in the control and experimental group.
○ : control group ; ■ : CMAFU ;
◆ : poly(CMAFU-co-MMA)

The average viability of the flies per culture treated with control group is 86% whereas the value is 0 %, 60 % and 0 % for experimental group I, II and III, respectively. It should be noted that *D. melanogaster* fed with 5-FU showed zero viability because of the toxicity of itself. Most of the flies treated with 5-FU were died in the early larva stages whereas the flies treated with poly(CMAFU-co-MMA) were died in their pupa stages. Meanwhile, of importance is also the fact that the viability data of CMAFU are much higher than those of 5-FU and copolymer.

3. 2. 2. Developmental time

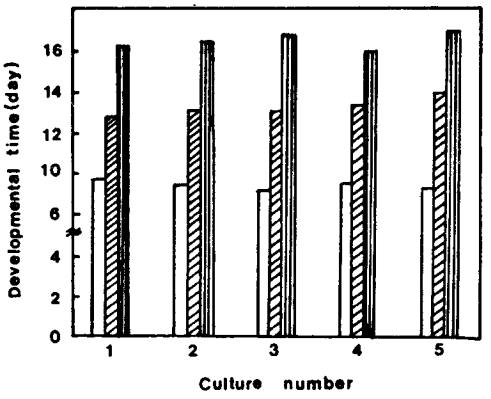


Fig. 4 Developmental time distribution in the control and treated groups.
□ : control group ; ▨ : CMAFU ;
▥ : poly(CMAFU-co-MMA)

Developmental time from egg to adult was calculated for that period during emergence after being cross, multiplied to total emerged flies of that day, after which summation was made and divided with total emerged flies.

Fig. 4 shows the growth rate of the flies treated with each experimental group. It is seen that the average developmental time is 9.43 days for the flies treated with control group whereas that is 13.29 days for experimental group II. The 5-FU and copolymer treated *D. melanogaster* showed no development since they were died in the early larva or pupa stages. It should be pointed that CMAFU showed a delay of the development and prolonged the developmental time 3 to 4 more days.

3. 3. 3. Fecundity

The fecundity was detected as the number of eggs laid per day per fly. The fecundity of OR female crosses in the F_1 generation is illustrated in Fig. 5. As shown in the fig, the relative values of the fecundity

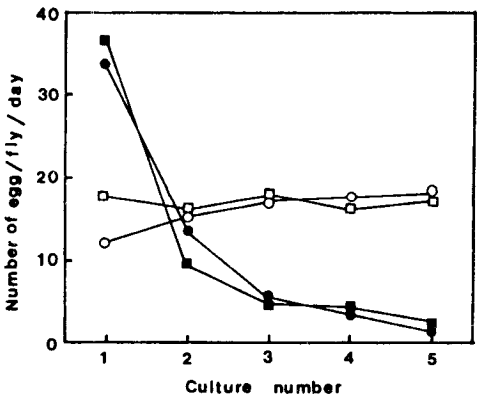


Fig. 5 Fecundity distribution in the control and treated groups.
○ : control group ; ● : 5-FU ;
■ : CMAFU ; □ : poly(CMAFU-co-MMA)

showed some fluctuation. This is caused by the fact that new females were supplied in 48 hrs intervals, in which the second day fecundity appeared normal.

The average fecundities appeared from the first to fifth cultures in which 16 eggs were in the control group, 12 eggs in the experimental groups I and II, and 17 eggs in the experimental group III. The fecundity of adults treated with control group increased slightly with repeated cultures whereas the fecundity decreased rapidly with cultures for experimental group II. There are no significant changes in fecundity for each culture in case of experimental groups III. The initial higher number of eggs per day on first culture for experimental group II may be attributed to the role of such chemicals to excite organs and accelerate fecundity temporarily.

Table 1. Productivity in the Control and Treated Groups

Group	Cultures				
	1	2	3	4	5
Control	197	276	300	318	301
5-FU	0	0	0	0	0
CMAFU	484	127	62	49	36
Poly(CMAFU-co-MMA)	10	3	7	3	7

Table 1 represents the productivity in the control and treated groups. The productivity is the number of total adult flies per each culture. The range of productivity is given as 0 to 606 for experimental group whereas that ranged from 197 to 318 for control group. The average productivity is 0, 152, 6 for experimental group I, II and III, respectively whereas that is 178 for control group. The average productivity of control group is almost constant per culture but that of other experimental group is remarkably decreased with cultures.

3. 4. Sex-linked recessive mutation

The next experiment was carried out to test recessive lethal mutation in the X-chromosome. Mating material *yf* ♀ =virgin females were sampled from ywmf & *yf* ♀ =strain. These experiments were conducted in the same procedure as in OR female crossing experiment, and were divided into control, experimental I, II and III groups. The control group was treated in 5% sucrose solution for 24 hr and five treated males were crossed with five *yf* ♀ =virgins. Four simultaneous replications were prepared for each experimental group.

After the 1st, 3rd, 5th, 9th, and 11th crosses, old females were discarded, new virgins were renewed, then they were daily transferred into a new vial unitil ten successive vials were obtained. With this mating, effect in successive stages of spermatogenesis was examined by the method suggested by Sankaranarayan and Sobels[15]. The results of egg laying were divided into four classes as follows : Brood A with 1 st to 4th successive vial, brood B with 5th to 8th, C with 9th to 10th, and D with 11th to 12th, respectively, It may be assumed that brood A, B, C and D derive mainly from treated mature sperm, spermatid, spermatocyte and spermatogonia, respectively.

In the next generation, males(XY) and females ($\hat{X}\hat{X}Y$) will be expected to emerge in a ratio of 1 : 1 if no lethal mutations occur on the treated X chromosome, since all flies with $\hat{X}\hat{X}\hat{X}$ and YY die during the development[16]. After crossing treated males

with mutant virgin females with attached X-chromosome, the sex ratio in F_1 is tested.

Table 2. The sex ratios(♂/♂+♀) of progenies from the crosses between yf : =female and 33 ppm treated OR males

Groups	No. of crosses	Total flies counted	Brood			
			A	B	C	D
Control	4	529	0.4818	0.5205	0.5977	0.5395
5-FU	4	840	0.5819	0.5906	0.5897	0.5827
CMAFU	4	673	0.5752	0.5629	0.5791	0.5816
Poly(CMAFU-co-MMA)	4	934	0.6161	0.5969	0.5952	0.5909

Sex ratio was measured as male numbers per total emerged progenies. Sex ratios by the broods in OR female crosses are represented in Table 2. In this table, males were treated with 33 ppm of each experimental group. Altogether, the total numbers of emerged adult flies ranged from 529 to 934. It can be seen that since the average sex ratio is ranged from 0.535 (control group) to 0.598(experimental groups) and all of each experimental group.

It was also shown that a significant difference in the sex ratio was not observed between the broods.

Inoue and Watanabe[16] reported on the sex-linked mutation for the males treated in 2.5×10^{-2} M of EMS when ten replication vials were prepared ; With total emerged 1221 flies, the sex ratio was observed to be 0.425. They pointed out that this relative value was a significant reduction and therefore EMS is found to induce mutation in X-chromosome.

4. CONCLUSIONS

The monomer, 1-(2-carbomethoxyacryloyl)-5-fluorouracil(CMAFU) was synthesized from trans-β-carbomethoxyacryl chloride. A copolymer of CMAFU with methyl methacrylate[poly(CMAFU-co-MMA)] was prepared with AIBN in cyclohexanone at 60 °C. The physiological genetic effect of 5-FU, CMAFU and its copolymer on *Drosophila melanogaster* was investigated by the adult feeding method of Lewis and Bacher.

1. The toxicity decreased in the order of 5-FU > poly (CMAFU-co-MMA) > CMAFU > control.
2. The average egg to adult viability of OR females was 86 % in the control group and 64 % for CMAFU. The *D. melanogaster* treated with poly (CMAFU-co-MMA) as well as 5-FU showed no viability because of their inherent toxicity.
3. The CMAFU delayed the developmental time of *D. melanogaster* for 3 or 4 more days.
4. The fecundity of adult females treated with CMAFU decreased rapidly with repeated cultures whereas that treated with control group increased slightly with cultures.
5. The sex-linked recessive mutation was not significantly affected by CMAFU and its polymers as well as 5-FU.

In conclusion, we found that the physiological genetic effects of CMAFU and its polymer on *Drosophila melanogaster* are considerably weaker than that of 5-FU.

ACKNOWLEDGMENTS

This work was financially supported by the Korea Science and Engineering Foundation. We thank Prof. Won-Ho Lee of Biology Dept. Pusan National University for his help in experiments on *Drosophila melanogaster* and useful comments on the manuscript. We also thank Mr. M. S. Shim for his helpful experimental assistance.

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