Effects of 5-Fluorodeoxyuridine and Related Halogenated Pyrimidines on the Sand-Dollar Embryo

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ABSTRACT

The embryo of the sand-dollar (*Echinarachnius parma*) was exposed to various concentrations of fluorinated pyrimidines immediately after fertilization. FUDR (5-fluorodeoxyuridine) was most active, and a concentration of 2 to 4 mγ/10 cc. (0.8 to 1.6 × 10^{-6} m.eq./liter) blocked development at the early blastula stage. Larger doses interrupted development at the same stage. This effect was prevented by thymidine (TDR) and thymine (T); and these pyrimidines protected against many times the minimal lethal concentration of FUDR. TDR was active as a protective agent if added just before early blastula formation.

The other fluorinated pyrimidines, 5-fluorouracil (FU), 5-fluorouridine (FUR), 5-fluorocytidine (FCR), 5-fluorodeoxycytidine (FCDR), and 5-fluoroorotic acid (FO), were also studied. These drugs produced effects on embryonic development similar to those seen with FUDR. The effective concentrations, however, varied greatly. T and TDR provided protection against these drugs, but in most cases they were not so effective as against FUDR.

5-Bromodeoxyuridine (BrUDR), beginning at the early blastula stage, caused a random pattern of embryonic death up to the pluteus stage. This drug has been shown to be incorporated into bacterial DNA. BrUDR protected embryos against the early lethal effects of FUDR presumably acting as a thymidine substitute, but the embryos died subsequently in a pattern similar to that seen with BrUDR alone.

FUDR and BrUDR appear to inhibit the formation and alter the structure of DNA, respectively, distinctive effects whch may provide a means for studying the role of DNA in embryonic development.

The development of the sand-dollar embryo (*Echinarachinus parma*) has been specifically blocked by the glutamine antagonists, DON (6-diazo-5-oxo-L-norleucine) and azaserine (o-diazoacetyl-L-serine) (24). These drugs have been shown to interfere with purine synthesis, by inactivating an enzyme involved in transferring an amino group to formylglycinamide ribotide (FGAR) to form formylglycinamidine ribotide (FGAM) (27). Glutamine is the natural substrate for this reaction. The effects of DON and azaserine could be prevented by the addition of physiological purines; the most active were guanine, hypoxanthine, and inosine.

5-Fluorouracil, a drug which specifically interfered with pyrimidine metabolism, was described by Heidelberger *et al.* (16) and Duschinsky *et al.* (8) in 1957, and subsequently the mechanism of action of 5-fluorouracil and related fluorinated pyrimidines was analyzed (3, 5, 6, 17, 18). Among other actions these drugs block the introduction of a methyl group into uracil to form 5-methyluracil (thymine), and 5-fluorodeoxyuridine (FUDR) appears to be most active and specific in this respect. The effects of FUDR and other halogenated pyrimidines, and the protective activity of natural pyrimidines were studied in the sand-dollar embryo.

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Materials and Methods

Mature sand-dollars are available during July and August in Salisbury Cove, Maine. Their development, although slower, parallels that of the sea-urchin embryo (15). Eggs and sperm were obtained by the potassium chloride injection method (33). The eggs were fertilized in a finger bowl by the addition of 5 drops of dilute sperm. The eggs were examined immediately after fertilization, and only the batches showing 95 per cent or more fertilization membranes were used. In most experiments 200 to 300 eggs were transferred to each compartment of a plastic ice-cube tray, each compartment containing 10 cc. of filtered sea water. The embryos were maintained at a temperature of 15°C. ± 1° in a thermostatically controlled refrigeration unit. They were examined at various intervals under a dissecting microscope until 72 hours after fertilization.

The chemicals used in these experiments were dissolved in filtered sea water and diluted to the appropriate concentrations. The solutions were made up fresh each day and the sea water was warmed to dissolve the poorly soluble drugs. The antimetabolites were added to the compartments containing the sand-dollar embryos, usually within 1 hour after fertilization, and the agents to be tested for protective action were added within 30 minutes. Instances in which the drugs were added at other times are noted in the text. The drugs were added to each compartment in volumes ranging from 0.1 to 1.0 cc., and the final concentrations are reported as mγ or gamma/10 cc. of sea water (the actual amount added) and as milliequivalents/liter. The great majority of experiments were performed two or more times.

The drugs used in this study, their abbreviations, and their sources are listed below.

Drug	*Abbreviation*
5-Fluorouracil	**FU** (*a*)
5-Fluorouridine	**FUR** (*a*)
5-Fluoro-2′-deoxyuridine	**FUDR** (*a*)
5-Fluorocytidine	**FCR** (*a*)
5-Fluoro-2′-deoxycytidine	**FCDR** (*b*)
5-Fluoroorotic acid	**FO** (*a*)
5-Bromo-2′-deoxyuridine	**BrUDR**
1-β-D-Xylofuranosylthymine	(*b*)
1-β-D-Ribofuranosylthymine (5-methyluridine)	(*b*)
1-β-D-Arabinofuranosylthymine ("spongothymidine")	(*b*)
Thymine	**T**
Thymidine	**TDR**
Uracil	**U**
2′-Deoxyuridine	**UDR**
Cytosine	**C**
Cytidine	**CR**
Cytidylic acid	**CRP**
Orotic acid	**OA**

We wish to thank (*a*) Hoffmann-LaRoche Inc. for samples of the fluorinated pyrimidines, and (*b*) Dr. J. J. Fox, of the Sloan-Kettering Institute for FCDR, and the abnormal pentosyl derivatives of thymine. The natural pyrimidines were obtained from commercial sources.

RESULTS

1. Normal Development of the Sand-Dollar Embryo:

At 15°C. *Echinarachnius parma* develops somewhat more slowly than other species that have been widely used in biological research (Table I). The first cleavage occurred at 80 to 90 minutes, a definite blastula appeared at 8 to 10 hours, and hatching occurred at 14 to 16 hours. At 20 hours gastrulation began, and by 24 hours the gut had formed about ⅓ of the way across the blastocoele. The gut was completed by 35 hours, and characteristic early plutei were present at 46 hours. The plutei grew and their arms elongated during the next 24 hours. These events proceeded, as described, in the vast majority of the untreated embryos, and are the reference points in describing and interpreting the effects of antimetabolites on development.

2. Effects of FUDR on Development:

FUDR was the most active of the fluorinated pyrimidines studied. The results with FUDR will be described in detail, and then compared with those obtained with the other halogenated pyrimidines.

(*a*) *FUDR Added Immediately before or after Fertilization.*—FUDR, even at high doses in excess of 4,000 gamma/10 cc. (1.63 m.eq./liter), had no detectable effects on fertilization or early development. The first evidence of toxicity appeared abruptly during the early blastula stage, about 8 hours after fertilization. In repeated experiments performed during the summer, there was a gradual increase in the amount of FUDR necessary to produce an abrupt interruption in development; the LD_{100} (24 hours) rose from 4 mγ/10 cc. in early July to 20 to 25 mγ/10 cc. in late August. A dose in excess of 25 mγ/10 cc. (1×10^{-5} m.eq./liter) consistently interrupted development at about 8 hours after fertilization. The embryos, in the early blastula stage at 8 hours, showed thickening of the blastula wall, which became translucent and homogenous in appearance. By 10½ hours after fertilization the blastocoele was virtually obliterated. Cytolysis then began, and by 11 hours only dark

TABLE I

Comparative Rates of Development of Echinoderm Embryos

	Arbacia (15) *punctulata* 23°C.	*Paracentrotus* (26) *lividus*	*Echinarachnius parma* 15°C.
First cleavage	50 minutes	71 minutes (18°C.)	93 minutes* (16°C.)
Blastula	6 hours	8 hours	8–10 hours
Hatched	7–8 hours	11 hours	14–16 hours
Early gastrulation	15 hours	16 hours	20 hours
Gastrulation complete	17 hours	19 hours	35 hours
Skeleton appears	16 hours	22–24 hours	23 hours‡
Early pluteus	20 hours	28 hours	40–46 hours
Pluteï	24 hours	40 hours	72 hours

* Personal communication, A. F. Rieck.

‡ Personal communication, G. Bevelander.

and disintegrating blastulae were present. The sequence of events remained constant despite variations in the concentrations of FUDR from 0.025 to 4,000 gamma/10 cc. (1.63 m.eq./liter to 1×10^{-5} m.eq./liter).

The lower concentrations of FUDR were more variable in their effects, and in many instances, particularly during early July, doses as low as 2 to 4 mγ/10 cc. interrupted development at the early blastula stage. Embryos exposed to concentrations lower than these showed a slightly thickened blastula wall, and free cells sometimes appeared in the blastocoele. The embryos often hatched at 14 hours, and moved actively. Gastrulation was usually blocked, but the abnormal blastulae sometimes continued to swim for as long as 36 hours. The estimated LD_{50} (48 hours) concentrations of FUDR (blocking approximately ½ the embryos before the pluteus stage) and LD_{100} (killing all embryos by 24 hours) for the months of July and August were as follows:

	LD_{100} (24 hrs.) mγ/10 cc.	LD_{50} (48 hrs.)
July, 1958	2–12	1–3
August, 1958	12–25	3–6

(*b*) *FUDR Added at Various Times after Fertilization.*—When FUDR was added at various dose levels at 0, ½, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, and 26 hours after fertilization, its toxicity remained unimpaired when the drug was added up to the 8th to 10th hour. When added in the range of the LD_{100} at 8 hours after fertilization, degeneration of the early blastulae occurred within a short time; the sequence of events being similar to those seen when FUDR was added shortly after fertilization. The blastula wall thickened by 9½ hours and cytolysis began at 15 hours. When FUDR was added 12 hours or later after fertilization 1 gamma/10 cc. was no longer acutely toxic, and 1,000 gamma/10 cc. added at 26 hours caused only a slight stunting in the plutei which developed.

(*c*) *FUDR Washed from Eggs at Various Periods after Exposure.*—Fertilized eggs were placed in sea water containing 50 mγ/10 cc. At ½, 1, 2, 4, 6, 8, and 12 hours after exposure 200 to 300 eggs were removed, placed in 10 cc. of sea water and lightly centrifuged; the supernatant fluid was removed, and fresh sea water added. The process was repeated 5 times, to be certain that all the free FUDR was removed. As a biological test for this, untreated fertilized eggs, were placed in the supernatant fluid from the last washing. They developed normally.

The embryos exposed and washed immediately were unaffected; those removed 30 minutes to 120 minutes later showed partial protection, although the dose of FUDR produced definite signs of toxicity; beyond 2 hours after the addition of FUDR there was no sign of protection. The concentration of FUDR was well above the LD_{100}; the protective effect of washing in the range of the LD_{50} dose was not determined.

When embryos were exposed to 50 mγ/10 cc. of FUDR 4 hours after fertilization, and the drug washed out at various times, partial protection was obtained in embryos washed up to 6 hours after fertilization. Embryos exposed at 8 hours were partially protected if the drug was removed within 15 minutes.

3. Protective Effects of Various Agents against Toxicity of FUDR:

(*a*) *Protective Agents.*—The naturally occurring pyrimidines were tested for their protective effects against FUDR. Embryos were exposed to various concentrations of FUDR shortly after fertilization, and the pyrimidines were added to the solution within 30 minutes. The results are shown in Table II. While UR and UDR (100 gamma/10 cc.) gave some protection against concentrations of FUDR greater than 0.256 gamma and less than 1.25 gamma/10 cc., 100 gamma/10 cc. of thymine and thymidine protected against the far higher concentration of 1,000 gamma/10 cc. of FUDR. The remarkable specificity of thymine and thymi-

TABLE II

Protective Action of the Physiological Pyrimidines against FUDR

FUDR	Pyrimidine concentration, 100 gamma/10 cc.					
	U	UR	UDR	T	TDR	C
gamma/10 cc.						
1000	0	0	0	+	+	0
50	0	0	0	+	+	0
5	0	0	0	+	+	0
1.25	0	0	0	+	+	0
0.256	0	+	+	+	+	0
0.031	0	+	+	+	+	0
0.016	0	+	+	+	+	0
0.008	0	+	+	+	+	0
0.004	0	+	+	+	+	0

0, No protection.

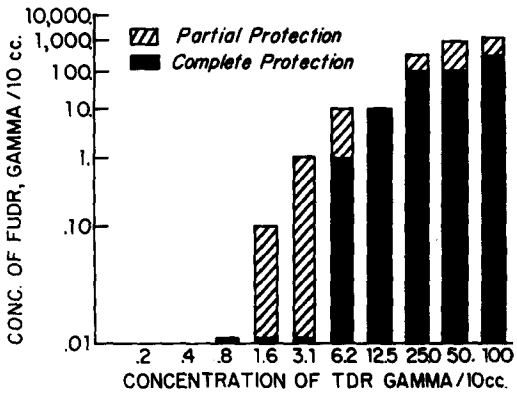
+, Partial to complete protection.

dine was confirmed in repeated studies, whereas slight and inconstant protective effects were observed with U, UR, and UDR.

The relationships between the protective concentrations of thymidine at various concentrations of FUDR were determined (Fig. 1). There is evidence of a competitive relationship at concentrations up to 100 gamma/10 cc. thymidine, but increasing the dose of thymidine above this provides only slight additional protection. Concentrations of thymidine below 0.80 gamma/10 cc. provided little protection. Thymine was somewhat less effective than thymidine. The maximum protective ratio of thymidine to FUDR was in the range of 1:20 to 1:40.

(*b*) *Protective Effects of Thymidine when Given at Various Periods after FUDR.*—Thymidine, 100 gamma/10 cc., was added at various times after fertilization to embryos exposed to a concentration of 100 mγ/10 cc. of FUDR at the time of fertilization. Thymidine, added up to 5 hours and 40 minutes after fertilization, gave complete protection, but after 6 hours it was no longer effective. Six hours after fertilization, embryos were exposed to 100 mγ/10 cc. FUDR; and then 100 gamma/10 cc. of thymidine was added at 5 minute intervals there-

5-Fluorouracil, **FU.** 5-Fluorouridine, **FUR.** 5 Fluoro-2′-deoxyuridine, **FUDR.** 5-Fluorocytidine, **FCR.** 5-Fluoro-2′-deoxycytidine, **FCDR.** 5-Fluoroorotic acid, **FO.** 5-Bromo-2′-deoxyuridine, **BrUDR.** 1-β-D-xylofuranosylthymine. 1-β-D-ribofuranosylthymine (5-methyluridine). 1-β-D-arabinofuranosylthymine ("spongothymidine"). Thymine, **T.** Thymidine, **TDR.** Cracil, **U.** 2′-Deoxyuridine, **UDR.** Cytosine, **C.** Cytidine, **CR.** Cytidylic acid, **CRP.** Orotic acid, **OA.**



TEXT-FIG. 1. Protective effect of thymidine against FUDR.

after. Complete protection occurred if the thymidine was added within 60 minutes; partial protection was found to 140 minutes afterwards, but beyond this period thymidine was no longer effective. This type of experiment was repeated, adding FUDR 8 hours after fertilization. Thymidine gave complete protection if added within 20 minutes, and partial protection was observed if the thymidine was added within 40 minutes; no protection was seen beyond this.

To further examine the specificity of the protective action of thymidine, three unnatural pentosyl thymidines (ribose, arabinose, and xylose) were tested for protective activity against FUDR, at a concentration of 100 gamma/10 cc. of the thymine analogue to 100 mγ/10 cc. of FUDR. Only thymine riboside exhibited protection.

4. Comparative Effects of Related Fluorinated Pyrimidines:

(*a*) *Fluorinated Pyrimidines Added Immediately after Fertilization.*—The toxic doses of the various fluorinated pyrimidines, in comparison with FUDR, are shown in Table III. The recorded doses were calculated on the basis of 5 or more separate experiments. Considerable variability in toxicity of these drugs was found from experiment to experiment and at different periods during the summer. The LD_{100} (24 hours) concentrations produced their effects at the early blastula stage in all instances; at much lower doses partial inhibition of development was sometimes observed at all stages up to the pluteus stage. In other experiments, however, the dose causing partial inhibition was about 50 per cent of the LD_{100} (24 hours), and at doses below this embryonic development was apparently

normal. The reasons for the variability in response are not known, but possibly nutritional factors in the embryo may be responsible.

The ribosyl and deoxyribosyl derivatives of cytosine (FCR, FCDR) were only slightly less active than FUDR. Fluorocytosine was not studied; FU and FUR were consistently less active, and FO was relatively ineffective. At the LD_{100} (24 hours) concentrations and above developmental disturbances appeared at 8 hours, and were identical with those seen with FUDR. In early experiments FUR was exceptional, however, in that concentrations of 250 to 1,000 gamma/10 cc. inhibited cleavage; the number of early divisions that occurred varied inversely with the concentration. Thymidine, uridine, and deoxyuridine did not prevent the effect of FUR on cleavage. Other fluorinated pyrimidines did not inhibit cleavage, and it was not observed with FUR later in the season.

TABLE III

Comparative Toxicity of the Fluorinated Pyrimidines

	LD_{100} (24 to 48 hours) mγ/10 cc.	m.eq./liter
5-Fluorouracil (FU)	50–200	$0.4–1.6 \times 10^{-4}$
5-Fluorouridine (FUR)	50–100	$2–4 \times 10^{-5}$
5-Fluorodeoxyuridine (FUDR)	2–4	$0.8–1.6 \times 10^{-6}$
5-Fluorocytidine (FCR)	3–12	$1–4 \times 10^{-6}$
5-Fluorodeoxycytidine (FCDR)	16–25	$0.6–1.0 \times 10^{-5}$
5-Fluoroorotic acid (FO)	50,000–250,000	$0.3–1.5 \times 10^{-3}$

(b) Protective Effects of the Natural Pyrimidines.—Using the same methods described above for FUDR, the natural pyrimidines, at an arbitrary concentration of 100 gamma/10 cc. were tested for protective activity against the fluorinated pyrimidines. The LD_{100} (24 hours) and LD_{50} (48 hours) concentrations of the fluorinated pyrimidines in the particular experiment described, and the concentrations of the fluorinated pyrimidines against which complete and partial protection were found are shown in Table IV. TDR was the most effective agent, and T was only slightly less active. Minor and inconstant protection was obtained with U and UDR against FUR, FUDR, and FCDR, and also with CR against FCDR.

The protective activity of TDR for the various fluorinated pyrimidines, based on the protection of 100 gamma/10 cc. of thymidine against multiples

TABLE IV

Protective Effect of the Physiological Pyrimidines against Various Doses of the Fluorinated Pyrimidines

Halogenated pyrimidines	LD_{100} (24 hrs.) gamma/10 cc.	LD_{50} (48 hrs.) gamma/10 cc.	Pyrimidines (100 gamma/10 cc.) Concentration (gamma/10 cc.) partial/complete protection							
			Thymine	Thymidine	Uracil	Uridine	Deoxyuridine	Cytidine	Deoxycytidine	Orotic acid
FU	0.100	0.050	135/10	500/31	0.5/None	None	None	None	None	None
FUR	0.125	0.030	31/0.5	62/1	None	0.5/0.25	0.50/0.25	None	None	None
FUDR	0.004	0.002	1000/100	+1000/1000	None	0.125/0.030	.125/0.030	None	None	None
FCR	0.050	0.012–0.025	100/0.5	100/10	None	.10/.05	None	0.050/None	0.050/None	None
FCDR	0.025	0.006–0.012	100/10	+100/100	None	.05/None	None	0.100/0.025	None	None
FO	250.0	62.0	+1000/1000	+1000/1000	250/None	250/None	None	None	None	None

TABLE V

Comparative Partial Protective Effects of Thymidine (100 gamma/10 cc.) against Multiples of the LD_{100} (24 Hour) of the Fluorinated Pyrimidines

	LD_{100} mγ/10 cc.	Multiples of LD_{100} against which partial protection occurred
FU	100	5,000
FUR	125	500
FUDR	4	+250,000
FRC	50	2,000
FCDR	25	+4,000
FO	250,000	+4

TABLE VI

Relation of Concentrations of Thymidine to Fluorinated Pyrimidines in Providing Complete Protection

Fluorinated pyrimidines	LD_{100} (24 hours)	Thymidine, gamma/10 cc.			
		400	100	25	2.5
		gamma/10 cc. of fluorinated pyrimidine against which complete protection occurs			
	gamma/10 cc.				
FU	0.300	1000	700	1.0	None
FUR	0.275	500	400	70.0	0.3
FUDR	0.015	1250	800	200.0	0.05
FCR	0.025	>100	90	3.0	0.04
FCDR	0.035	>100	100	5.0	None

of the LD_{100} (24 hours) for each drug, is given in Table V.

Only preliminary attempts were made to determine protective ratios of TDR to the various fluorinated pyrimidines (Table VI). The study was handicapped because supplies of FCR and FCDR were inadequate. The complete protective activity of TDR was maximal at concentrations of 100 to 400 gamma/10 cc. and above. Between 25 and 100 gamma/10 cc. the protection was directly related to the concentration of TDR. Below 25 gamma/10 cc. the complete protective activity of TDR dropped rapidly, and was minimal at 2.5 gamma/10 cc.

5-Fluorouracil, **FU.** 5-Fluorouridine, **FUR.** 5 Fluoro-2′-deoxyuridine, **FUDR.** 5-Fluorocytidine, **FCR.** 5-Fluoro-2′-deoxycytidine, **FCDR.** 5-Fluoroorotic acid, **FO.** 5-Bromo-2′-deoxyuridine, **BrUDR.** 1-β-D-xylofuranosylthymine. 1-β-D-ribofuranosylthymine (5-methyluridine). 1-β-D-arabinofuranosylthymine ("spongothymidine"). Thymine, **T.** Thymidine, **TDR.** Uracil, **U.** 2′-Deoxyuridine, **UDR.** Cytosine, **C.** Cytidine, **CR.** Cytidylic acid, **CRP.** Orotic acid, **OA.**

5. Effects of 5-Bromodeoxyuridine on Development:

a) Toxicity.—In contrast to the abrupt toxic effects of LD_{100} (24 hours) concentrations of the fluorinated pyrimidines, BrUDR was gradually lethal to the culture, so that mixed populations of dead, disintegrating, and developing embryos were found at all periods after the blastula stage. Nevertheless BrUDR, at appropriate doses, was ultimately lethal to all the embryos.

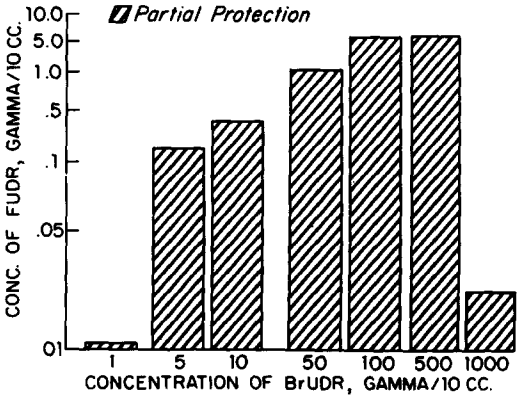
It was difficult to obtain a sharp LD_{100} of BrUDR. Following fertilization the effective concentrations producing embryo death gradually diminished from over 1,000 gamma/10 cc. at 24 hours to 1 to 5 gamma/10 cc. at 72 hours. Concentrations of 1,000 gamma/10 cc. applied immediately after fertilization rarely caused embryonic deaths until after 8 hours. After this, dead and dying embryos appeared, and at 24 hours, at a dose

TABLE VII

Protective Activity of the Physiologic Pyrimidines against BrUDR

Conc. BrUDR	Pyrimidines, 100 gamma/10 cc.							
	U	UR	UDR	C	CR	T	TDR	OA
gamma/10 cc.	Estimated protection*							
1000	0	0	0	0	0	±	±	0
500	0	+	0	0	0	+	+	0
125	0	+	+	±	+	+	+	0
62.5	±	+	+	+	+	+	+	0

* 0, none.
±, slight.
+, definite.



TEXT-FIG. 2. Protective effect of BrUDR against FUDR.

of 1,000 gamma/10 cc. (3.25×10^{-1} m.eq./liter) about 35 per cent of the embryos were dead, 40 per cent showed various stages of inhibition, and the remainder appeared normal. These surviving embryos often proceeded to the pluteus stage, and although indistinguishable from the controls in appearance, died between 40 and 70 hours. The dose permitting some survivors after 72 hours is below 1 gamma/10 cc. At 20 gamma/10 cc. a number of embryos advanced to the pluteus stage before dying, while 50 gamma/10 cc. was usually lethal at 48 hours.

(*b*) *Protective Effects of the Physiologic Pyrimidines.*—Because of the gradual lethality of BrUDR, it is difficult to define a clear quantitative protective relationship between the natural pyrimidines and BrUDR. In Table VII the degree of protection is estimated at 24 hours after fertilization. T and TDR show the greatest protective activity, but all the physiologic pyrimidines gave some degree of protection except orotic acid. The protection was not complete, and the embryos in these experiments were all dying at 48 hours. High concentrations of TDR, up to 5,000 gamma/10 cc. given shortly after fertilization, gave partial protection against 1,000 gamma/10 cc. of BrUDR, and some of the plutei were surviving at 72 hours.

6. Protective Effect of BrUDR on Embryos Treated with FUDR:

BrUDR, added to embryos exposed to supralethal concentrations of FUDR, caused definite but temporary protection, and development proceeded beyond 11 to 12 hours after fertilization (Fig. 2). Most of the embryos remained viable for some time after 15 to 16 hours, and some continued to develop to form gastrulae and plutei. By 72 hours, however, all of the embryos were dead. Even embryos exposed to 100 gamma/10 cc. of FUDR showed a definite prolongation of survival on BrUDR.

When observed at 24 hours, concentrations of 5 to 500 gamma/10 cc. of BrUDR were still producing definite temporary protection against concentrations of 6.25 gamma/10 cc. of FUDR. The embryos were not all normal, in that many were dead or dying in a pattern similar to that seen with BrUDR alone, but also there were numerous motile survivors. Survivors at this stage were never seen at these concentrations of FUDR alone. At 44 hours after fertilization, 5 to 100 gamma/10 cc. of BrUDR was still producing conspicuous protection against 25 to 400 mγ/10 cc. of FUDR, and some of these embryos survived to 72 hours. In more detailed experiments 5 to 10 gamma/10 cc. of BrUDR gave partial but definite protection against 20 to 200 mγ/10 cc. of FUDR, with some embryos surviving to 72 hours.

FUDR, 200 mγ/10 cc., was added 8 hours after fertilization, and BrUDR (10 gamma/10 cc.) was added at 10 minute intervals thereafter up to 2 hours. Some transient protection occurred if BrUDR was added within 40 minutes, but not beyond this time suggesting an effect similar to that seen with thymidine.

DISCUSSION

By interfering with the methylation of uracil nucleotide or nucleoside, the fluorinated pyrimidines inhibit the synthesis of thymidylic acid and thereby block DNA synthesis (9, 16). This is their major action and FUDR is particularly specific in producing this effect. The fluorinated pyrimidines have several other distinctive actions (3–6, 17, 18, 25, 31). They may be incorporated into RNA to produce an unnatural or "fraudulent" RNA (3, 5) and inhibit the conversion of uracil or orotic acid into RNA uracil. They also may be involved in the formation of fluorine-containing pyrimidine nucleotides and coenzymes (32). In human cells growing in tissue culture (11, 32), whereas TDR will protect against FUDR and FCDR, it does not reverse the inhibitory effects of FU, FUR, and FCR. Thus, the biological effects of each of these drugs, and the protective value of the physiological pyrimidines vary from system to system, *in vivo* and *in vitro*, differences presumably being due to specific actions of the chemical, the sensitive components of the system, the rate of penetration of the drug, and its metabolism in the host and within the cell. While this is a problem of major interest, it does not seem to be immediately relevant to effects of fluorinated pyrimidines on the sand-dollar embryo.

These compounds appear to have a uniform effect on development, an effect that is efficiently prevented by T and TDR. This suggests that the lethal action of these agents in the sand-dollar embryo is due to the block in the methylation of uracil. While TDR gives the greatest protection against FUDR, and its protective activity varies quantitatively among the other fluorinated pyrimidines (Table IV), it is highly active in all instances. These agents at supralethal doses probably block

or modify other vulnerable systems, which are able to express themselves when the TDR deficiency is corrected. An adequate quantitative study of the protective effects of the physiological pyrimidines, alone and in combination, against the various fluorinated pyrimidines has not been completed.

It is worth noting that the pyrimidines are highly specific in their actions in the sand-dollar, and this organism may be used to analyze for specific enzymatic conversions. For example, T is about ½ as active as TDR by weight in protecting against FUDR. It presumably forms TDR before it is incorporated into DNA. Thymine riboside also protects against FUDR. It may conceivably be incorporated directly into DNA as a thymine substitute, but more likely the ribosyl group is removed and T then goes to TDR. The xylose and arabinose derivatives of T are inactive. Fox *et al.* (13) have shown that an extract of *E. coli* B will cleave 5-methyluridine, but not the 1-β-D xylo-, lyxo- or arabino- derivatives of thymine. Presumably the embryo is also unable to cleave these abnormal derivatives to give usable thymine.

It is assumed that the fluorinated pyrimidines produce a TDR deficiency in the embryo, and the results with FUDR will be used to illustrate the discussion. At multiples of the LD_{100} (24 hour) concentrations, or at the minimum effective dose, FUDR does not seem to affect the embryo until the early blastula stage, about 8 hours after fertilization. The significance of this hour is supported by several findings: (1) when TDR is added as late as 6 hours after the fertilized embryos are exposed to FUDR, TDR will provide protection; (2) when FUDR is added 8 hours after fertilization it will promptly block development; and (3) when FUDR is added at 8 hours, TDR must be added within 20 to 40 minutes to give protection. Why is the early blastula stage so critical in responding to the lethal effect of FUDR?

Many of the events of embryonic development in the echinoderm have been described in detail (14, 15, 26). In *P. lividus*, Elson *et al.* (12) have shown that there are relatively small changes in RNA content during development; a transient fall occurs after fertilization, a rise during gastrulation and a decrease during the pluteus stage. There is, on the other hand, a steady increase in the DNA content of the embryo, from a barely detectable amount at fertilization to approximately 7.1 mγ/embryo at the pluteus stage. Before fertilization the egg has an excess of DNA, but by the 16 cell stage the DNA content corresponds to the number of diploid cells. At 10 hours the estimated number of cells, in the embryo, is 1000 (29), and the DNA content is equivalent to 900 diploid cells; and at 40 hours the number of cells is estimated as 2000 to 3000, and the DNA content indicates 3,550 cells. The cells appear to double every hour up to 10 hours after fertilization; beyond this time each cell needs to divide only once, or twice at the most to complete the number of cells in the pluteus.

The echinoderm embryo appears to have, not only an excess of DNA at fertilization, but also DNA precursors which can tide it over until the *de novo* synthesis of purines and pyrimidines begin. Hoff-Jorgensen and Zeuthen (21) described cytoplasmic deoxynucleosides in high concentration in the sea-urchin egg. Abrams (1), using glycine C^{14} in tracer studies, concluded that the larger part of DNA purines in the sea-urchin embryo is probably derived from unknown endogenous precursors. Hultin (22) and Hultin and Wessel (23) found that the incorporation of C^{14} formate into the purines of the sea-urchin embryo is low during the early period of development, but rapidly increases during the early blastula stage. In a recent study, Bieber (2) described large stores of deoxyribosides in the cytoplasm of the frog egg. The existence of purine and pyrimidine deoxyribosides in the cytoplasm is presumably sufficient, in the presence of a block in TDR formation by FUDR, to sustain the embryo to the early blastula stage. At this time, presumably the acute TDR deficiency is lethal to the embryo within 20 to 40 minutes. The addition of TDR presumably bypasses the block, and the embryo is able to proceed normally. The toxic effect of FUDR is considerably diminished when it is added 12 or more hours after fertilization. Presumably cell division and DNA synthesis per cell is diminished at this time, and the embryo is not vulnerable to FUDR. It would be important, however, to determine if plutei surviving the high concentrations of FUDR at 12 to 16 hours are deficient in cell number and DNA content, indicating that FUDR is still blocking TDR synthesis without killing the embryo.

The glutamine antagonist, 6-diazo-5-oxo-L-norleucine (DON), which interferes with purine syn-

5-Fluorouracil, **FU.** 5-Fluorouridine, **FUR.** 5 Fluoro-2′-deoxyuridine, **FUDR.** 5-Fluorocytidine, **FCR.** 5-Fluoro-2′-deoxycytidine, **FCDR.** 5-Fluoroorotic acid, **FO.** 5-Bromo-2′-deoxyuridine, **BrUDR.** 1-β-D-xylofuranosylthymine. 1-β-D-ribofuranosylthymine (5-methyluridine). 1-β-D-arabinofuranosylthymine ("spongothymidine"). Thymine, **T.** Thymidine, **TDR.** Uracil, **U.** 2′-Deoxyuridine, **UDR.** Cytosine, **C.** Cytidine, **CR.** Cytidylic acid, **CRP.** Orotic acid, **OA.**

thesis, has a somewhat similar effect on the sand-dollar embryo. DON is effective at 2 to 4 mγ/10 cc. of sea water (1.17 to 2.34 × 10^{-6} m.eq./liter) which is in the same range as FUDR (24). At minimal to many times the lethal dose, the first evidence of DON toxicity occurs at 14 hours, shortly after hatching. The cells begin to cytolyze, and the blastulae disintegrate. At lower doses the embryos may advance to early gastrulation before the block appears. Although the physiological purines are protective, the protective level is not great. Despite the amount of the normal purine added, it has not protected at concentrations greater than 30 times the minimum effective dose of DON. It is suggested that larger concentrations of DON block metabolic processes not related to purine synthesis. Eidinoff *et al.* (10) *in vivo* and in tissue slice experiments have shown that DON depresses the incorporation of ureidosuccinic and orotic acid into the cytosine moiety of the nucleic acids. The longer period of embryonic development after DON as compared to FUDR suggests that the embryo has more substantial reserves of purine than pyrimidine precursors.

The TDR deficiency produced by FUDR suggested the possibility of testing an abnormal TDR substitute for its ability to protect the embryo against FUDR, and its effect on embryonic development. Hitchings *et al.* (19, 20) found, among other complicated relationships, that 5-bromouracil inhibited the growth of *L. casei*, and this effect was prevented by thymine. 5-Nitrouracil also inhibited bacterial growth, but 5-bromouracil partially restored it. It was subsequently shown that 5-bromouracil is incorporated into DNA as a thymine substitute (7, 34) and it then inhibits bacterial growth (30). The incorporation of metabolic analogues into cellular components has been reviewed recently by Matthews (28).

We have shown that BrUDR has a marked inhibitory effect on the sand-dollar embryo. It is relatively non-toxic until the early blastula stage, and then the embryos begin to die off randomly at advancing stages of development. BrUDR is presumably incorporated into DNA, and it was shown that the physiological pyrimidines are relatively poor in protecting against BrUDR. The embryo possibly does not incorporate BrUDR early in development, or more likely it is not dependent on intact DNA at the early stage of development. As a result of BrUDR incorporation after the early blastula stage, embryonic death occurs randomly possibly when (1) mutations are induced in various critical cells, which express their lethal effects at different stages of development, (2) a critical amount of BrUDR is taken up by the embryonic DNA, or (3) a particular cell or group of cells in the embryo are damaged by BrUDR. The relation of the location and extent of thymidine replacement in embryonic DNA to the embryonic death is an interesting problem.

One possible explanation of the ability of BrUDR to protect the embryo temporarily against FUDR is that BrUDR is acting as a TDR substitute. BrUDR appears to be less active than TDR by a factor of 10–20. The problem of interpreting the quantitative protective activity of BrUDR is difficult, however. If too small a concentration of BrUDR is used, protection does not occur. An excessive dose of BrUDR can be lethal by itself early in the blastula stage, and this effect may be enhanced if the incorporation of BrUDR were possibly increased in the presence of FUDR. When the proper concentrations of BrUDR are given to FUDR-treated embryos, development will proceed beyond the point of FUDR toxicity, but subsequently the embryos invariably succumb. It is suggested that the abnormal DNA produced by BrUDR is capable of sustaining embryonic development and differentiation, but finally, for possible reasons noted above, the deficient DNA is no longer competent.

It will be necessary to demonstrate in the echinoderm embryo that DON is blocking purine synthesis and inhibiting DNA formation, that FUDR is producing TDR deficiency, and that BrUDR is incorporated in large amounts into DNA. The technical methods are available to accomplish this. These highly specific drugs, which presumably are acting in a precise manner to modify the synthesis of nucleic acids and the structure of DNA, may prove to be delicate tools to establish the role of nuclear function in the development of the echinoderm embryo.

CONCLUSIONS

1. 5-Fluorodeoxyuridine (FUDR), a drug which interferes with the methylation of uracil to form thymine, specifically interrupted the development of the sand-dollar embryo (*Echinarachnius parma*) during the early blastula stage. It was effective in concentrations of 2 to 4 mγ/10 cc. sea water (0.8 to 1.6 × 10^{-6} m.eq./liter).

2. Thymidine (TDR) and thymine (T), produced a high level of protection against FUDR, even when added just prior to the early blastula

stage. The relationship between FUDR and TDR is not strictly competitive; 100 gamma/10 cc. of TDR will protect against 500 gamma/10 cc. of FUDR. The other physiological pyrimidines show a low or negligible protective activity.

3. Other fluorinated pyrimidines, 5-fluorouracil, 5-fluorouridine, 5-fluorocytidine, 5-fluorodeoxycytidine and 5-fluoroorotic acid also produce effects on the sand-dollar embryo similar to FUDR. The effective concentrations vary, but, in all cases, they are higher than FUDR. Thymidine provides effective protection against all the fluorinated pyrimidines, but the degree of protection varies for each drug.

4. 5-Bromodeoxyuridine will cause random deaths of the embryos at all stages of development beyond early blastula formation. The physiological pyrimidines provide slight, but definite protection against BrUDR. BrUDR will temporarily protect the embryo against supralethal doses of FUDR, suggesting that it is acting as a TDR substitute. Ultimately the embryos die in a pattern similar to that seen with BrUDR alone.

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