of leukemic patients upon giving test doses of folic acid 6-10 days after aminopterin administration and has observed an increase in the excretion of a material that would support the growth of *Streptococcus faecalis*. She has interpreted her data to suggest a displacement by the antagonist. Thus, in agreement with Swenseid and Nichol, the present direct finding of a 3-5 fold increase in urinary CF activity in man after methotrexate administration probably reflects a displacement of CF by the drug.

Summary. *L. citrovorum* #8081/A of Hutchinson's laboratory has been shown to be an appropriate microorganism for measurement of urinary CF activity under the experimental conditions described. A 3-5 fold increase of urinary CF activity was repeatedly observed in subjects receiving large doses of methotrexate (Amethopterin). The possible sources of this increased urinary CF activity were discussed.

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1. Li, M. C., Hertz, R., Spencer, D. B., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 361.

2. Nichol, C. A., Welch, A. D., *ibid.*, 1950, v74, 403.

3. Greenspan, E. M., Goldin, A., Schoenbach, E. B., *Cancer*, 1950, v3, 856.

4. Hutchinson, D. J., Burchenal, J. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 516.

5. Silverman, M., Gardiner, R. C., *J. Bact.*, 1956, v71, 433.

6. Swenseid, M. E., Swanson, A. L., Miller, S., Bethell, F. H., *Blood*, 1952, v7, 302.

7. Sauberlich, H. E., Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

8. Keresztesy, J. C., Silverman, M., *J. Am. Chem. Soc.*, 1951, v73, 5510.

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Effects of DON (6-Diazo-5-Oxo-L-Norleucine) and Azaserine on the Sand-Dollar Embryo.* (23639)

DAVID A. KARNOFSKY AND GERRIT BEVELANDER

Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Department of Histology, College of Dentistry, New York University, and Mt. Desert Island Biological Laboratory, Salisbury Cove, Me.

The antimetabolites, DON (6-diazo-5-oxo-L-norleucine) and azaserine (0-diazo-acetyl-L-serine), are highly toxic, and often produce developmental abnormalities in chick(1,2), rat(3-5) and dog(6) embryos. It was of interest to examine the effects of these agents on echinoderm embryos, since there are many studies on the biochemical events associated with their development(7-9). Sand-dollar embryos (*Echinarchius parma*) are available in quantity during July and August in Salisbury Cove, Me., and their development, while slower, parallels that of the more extensively studied sea-urchin embryo.

Materials and methods. Eggs and sperm were obtained from sand-dollars by the KCl-injection method(10). The eggs were fertilized in a finger-bowl by adding 5 drops of dilute sperm. In most experiments 200 to 500 fertilized eggs were then transferred to compartments of plastic ice-cube trays, each compartment containing 10 cc of filtered sea-water. The trays were floated on circulating fresh sea-water, so that the embryos were kept at the temperature of the sea, 13-15°C. The embryos were examined under a dissecting microscope at regular intervals up to 72 hours after fertilization. At 36 hours, 5 cc of fresh sea-water was added to each compartment to replenish the oxygen supply. The chemicals used were dissolved in filtered sea-water; appropriate dilutions were made in sea-water from stock solutions which were prepared

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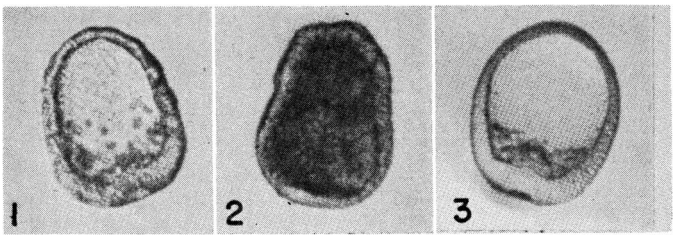

FIG. 1. Sand-dollar embryos, 22 hr after fertilization: 1. Control, early gastrulation. 2. Embryo treated with 8 mγ DON/10 cc blocked in late blastula stage. Blastula cavity contains dissociated cells, cytolyzing. 3. Embryo, treated with 64 mγ DON and 32 γ inosine/10 cc seawater. Apparently normal early gastrula.

each day. The sea-water was warmed in order to dissolve the poorly soluble compounds. The drugs used were DON, azaserine, adenine, adenosine, yeast adenylic acid, guanine, guanosine and sodium guanylate, xanthine, hypoxanthine, inosine, adenine 1-N oxide (11), DPN, ATP, AIC (4-amino-5-imidazolecarboxamide), glutamic acid, cytosine, thymine and uracil. DON was the principal agent used, and several experiments for purposes of comparison were performed with azaserine. The antimetabolites were added to the sand-dollar embryos within 1 to 2 hours after fertilization, and the chemicals tested for protective activity were added 1 to 2 hours later, unless stated otherwise. The drugs were added in a volume of 0.25 cc of sea-water in most cases. The concentration of the test chemicals to which the embryos were exposed is expressed as milligamma (10^{-9} g) or gamma (10^{-6} g)/10 cc of sea-water. Most experiments were performed at least 2 times, and many of the experiments, initially run in the summer of 1956, were repeated in 1957.

Results. Normal development of the sand-dollar embryo. The sequence of development of *Echinarchius parma* has not received the detailed attention accorded the sea-urchin egg (9). In this study the embryos usually were examined at 8-10 hours, 22-24 hours, 46-48 hours and 72 hours after fertilization. Cleavage begins within 1 to 2 hours following fertilization; at 2½ hours the embryos are usually in the 8-cell stage. At 8 to 10 hours a *definite blastula* has formed, movement begins around 14 hours, and the embryo hatches at 14 to 16 hours. At 22 hours early *gastrulation* has begun (Fig. 1-1), at 30 to 36 hours the gut has almost completely formed, at 46 hours the *pluteus stage* is beginning, and at 72 hours well-formed plutei are present. These events proceeded consistently in the great majority of untreated embryos.

I. *Effects of DON on Development.* a) *DON added shortly after fertilization. One hundred to 1,000,000 milligamma of DON/10 cc of sea-water.* These doses had no important effect on cleavage, although slight retardation was noted at the highest dose. Ten hours after fertilization apparently normal blastulae were present, and at 14 hours these hatched and began to rotate. At the highest doses cytolysis began at this time, and at 22 hours the blastulae were degenerating. Even at the lowest dose, the embryo did not develop beyond the blastula stage, although some assumed a pointed appearance, suggesting early gastrulation. In some cases swimming persisted until 48 hours before the embryo disintegrated. The invariable effect at 100 milligamma/10 cc and higher doses was apparently normal development to the blastula stage, and then cytolysis and degeneration.

Sixteen to 64 milligamma/10 cc sea-water. Eight separate experiments in this dose range gave consistent results. The embryos proceeded to the blastula stage, and many were interrupted and disintegrated in the next 24 hours. At the lowest doses occasionally the embryos assumed an elongated appearance, and gastrulation began, but the mesenchyme was disorganized, filled the early gastrula cavity, and the embryo finally degenerated. In some cases the degenerating embryos continued to rotate for 48 hours, and the skeleton formed. The minimum concentration of

TABLE I. Effects of Various Doses of DON on Development.

Dose, mγ/10 cc	Total No. exp.	No. experiments showing— Interruption in gastrulation	Gastrulation, but short-armed plutei	Retardation in development of plutei
8	10	7	2	1
4	10	3	5	2
2	10	2	2	6
1	3	0	2	1

DON invariably producing a developmental block in the blastula or early gastrula stages was 16 milligamma/10 cc of sea-water (9.3×10^{-9} M).

One to 8 milligamma/10 cc sea-water. Doses of 1 to 8 milligamma of DON/10 cc sea-water produced definite retardation of development (Fig. 1-2) but the results were not as consistent as those seen at 16 milligamma/10 cc (Table I). The dose calculated to produce consistent developmental retardation at the early gastrula stage in at least 50% of the experiments is in the range of 2 to 4 milligamma/10 cc of sea-water ($1.17\text{-}2.34 \times 10^{-9}$ M).

b) *DON added at various times after fertilization.* In 2 separate experiments DON was added at 3, 7, 12 and 20 hours after fertilization. DON added up to 7 hours, exhibited unimpaired activity in blocking gastrulation. When added at 12 hours, 8 milligamma 10 cc still produced a definite block in gastrulation, but the embryos survived longer than the embryos treated earlier with DON. DON appeared to lose much of its effect on gastrulation when added at 20 hours. At doses of 64 milligamma/10 cc and higher, gastrulation proceeded to completion, but development stopped in the early pluteus stage and cytolysis occurred. At doses below 32 milligamma/10 cc pluteus formation was retarded and mixed cultures of blocked and advanced plutei were found. When DON was added to swimming plutei at 72 hours, 102,400 milligamma/10 cc of sea-water did not appear to interfere with their activity during the next 24 hours.

c) *DON added prior to fertilization.* Sand-dollar eggs and sperm were placed in separate dishes of sea-water containing DON, 102,400 milligamma/10 cc. After 1 hour exposure the eggs and sperm were mixed in separate dishes containing the same concentration of DON in the following combinations: 1) treated eggs, normal sperm, 2) normal eggs, treated sperm, 3) treated eggs, treated sperm, and 4) normal eggs and normal sperm. In all dishes fertilization, cleavage and blastula formation proceeded in an apparently normal manner. The dishes treated with DON were all blocked in the blastula stage, the controls developed normally. It is concluded that DON does not interfere with the function of the sperm or egg in the process of fertilization, nor the subsequent cleavage.

d) *DON washed from eggs at various periods after exposure.* Fertilized eggs were placed in 50 cc of sea-water containing 320 milligamma DON (64 milligamma/10 cc). At 10, 40, 90, 165, 315, 450 and 690 minutes after exposure, 500-1000 eggs in 1 cc of fluid were removed and placed in 10 cc sea-water. The eggs were centrifuged lightly, and the supernatant fluid decanted so that less than 1 cc remained. Ten cc sea-water were again added, the eggs centrifuged, and the supernatant fluid decanted. The embryos were then placed in 10 cc sea-water and development allowed to proceed. The original solution had 64 milligamma/10 cc sea-water, and the washing procedures diluted it at least 1000 times; thus, the maximum concentration of DON in the final medium in which the embryo developed should have been less than 0.064 milligamma/10 cc sea-water. To demonstrate this, untreated fertilized eggs were placed in the supernatant fluid from the last washings; development proceeded normally. Control, washed and centrifuged embryos also showed normal development.

The results on the washed eggs were as follows: washed at 10 min.—normal plutei developed at 72 hr (complete protection). 40 min.—normal plutei developed at 72 hr (complete protection). 90 min.—at 72 hr blocked gastrula and plutei (moderate protection). 165 min.—blocked gastrula and plutei (moderate protection). 315 min.—mostly blastulae and blocked gastrula, a few early plutei

TABLE II. Summary of Observations on Protection Provided by Purines, Pyrimidines and Related Agents against DON in Sand-Dollar Embryo. (Maximum conc. of protective agent tested, 128 γ/10 cc.)

Substance tested	Protective action	Conc. DON, mγ/10 cc — Degree of protection — None	Slight	Almost complete	Min effective dose protective agent, γ/10 cc
Guanine	+	256	128	32- 64	2
Guanosine	+		32-64	8- 16	*
Sodium guanylate	+		32-64	8- 16	*
Adenine	+	256	128	32- 64	16-32
Adenosine	+		64	16- 32	*
Yeast adenylic acid	+		32-64	16	*
Xanthine	+	256	64	16- 32	*
Hypoxanthine	+	256	128	32- 64	1-2
Inosine	+	512	256	64-128	2
Adenine oxide	—	4			
DPN	+		64	16- 32	16-32*†
ATP	+		32-64	8- 16	*
AIC	—	2			
Glutamic acid	—	2			
Cytosine	—	2			
Thymine	—	2			
Uracil	—	2			

* Not titrated for minimum effective dose. † Swimming movements blocked.

(poor protection). 450 min.—almost complete interruption in early gastrulation, rare plutei (poor protection). 690 min.—all died in blocked blastulae stage. It thus appears that if the DON is removed up to 40 minutes after exposure complete protection occurs; from 90-165 minutes there is moderate protection; 5 to 7½ hours, definite but poor protection and after 11½ hours, no protection was observed.

II. *Agents protecting against the toxicity of DON.* a) *Protective effects of various agents.* In protection studies, usually the protective agent was given at 32 γ/10 cc, and DON at doses of 128, 64, 32, 16 and 8 milligamma/10 cc of sea-water; thus, the dosage ratios are in the range of 250-4000:1. These experiments were repeated a number of times, with several variations in dosage, and the following consistent results were obtained (Table II). The most active protective agents were *guanine, inosine* and *hypoxanthine.* Guanine and inosine were tested at minimal doses, and 2-4 γ partially protected against 64-128 milligamma DON/10 cc (Fig. 1-3). Increasing the dose of guanine and inosine did not protect against higher concentrations of DON. Adenine and xanthine were less active, and the ribosides and ribotides of adenine and guanine were less effective than adenine but gave some protection against doses of 32 to 64 milligamma of DON. DPN and ATP also gave definite protection. It is of interest that DPN paralyzed the swimming movements of the embryos, and although development proceeds for a time, the plutei were clumped at the bottom of the dish. When DPN is added to untreated motile gastrulae or plutei, 32 γ/10 cc stopped movement within 1 minute. Adenine oxide, AIC, glutamic acid, cytosine, thymine and uracil gave no appreciable protection against DON. At the doses used, the agents examined for protective activity, with the exception of DPN, appeared to have no adverse effect on the development of the embryo.

b) *Protective effects of inosine when given at various periods after DON.* Inosine, 32 γ, was added to embryos exposed to doses of DON ranging from 16 to 4096 milligamma/10 cc. DON was added 1 hour after fertilization, and inosine was given either 20 minutes before or after DON. Inosine had similar protective activity when given before or after DON; there was little protection at 256, slight at 128, and complete protection at 64 milligamma DON/10 cc. Embryos, exposed to various doses of DON (64, 32, 16 milligamma/10 cc) shortly after fertilization, were treated subsequently (3½, 6, 9, 12 and 20 hours after ferti-

lization) with inosine (32 γ/10 cc). Inosine provided protection against DON when added up to 12 hr. Protection was poor at 20 hr, where evidence of blastula interruption was present before inosine was added. Embryos exposed to lower doses of DON (16 milligamma/10 cc) and treated with inosine at 20 hours sometimes gastrulated, but were blocked in the early pluteus stage, suggesting that slight protection was possible even at 20 hours.

III. *Azaserine (Diazo-L-Serine).* Azaserine, an analogue of DON, was compared with DON for its effects on the sand-dollar embryo. Interruption in the blastula stage was produced by 500 milligamma/10 cc, more advanced development with interruption in gastrulation occurred at 200-250 milligamma/10 cc, and mixed development, blocked gastrulae and plutei at 100-128; normal plutei were found among embryos treated at 64 milligamma. Thus, the effects produced by 500, 256 and 128 milligamma of azaserine/10 cc of sea-water were equivalent to those of 16, 8 and 4 milligamma of DON, an activity ratio of approximately 32:1. In protection studies guanine at 32 γ/10 cc gave slight protection at 2560 to 5120 milligamma of azaserine/10 cc; definite protection at 1280, and complete at 640. Adenine was less active. The protection ratios are thus not related to the amount of the azaserine or DON used, but to the multiples of the minimum effective doses of DON and azaserine.

Discussion. The effects of DON and azaserine on the sand-dollar embryo are apparently similar, but DON is approximately 32 times more active by weight. The data suggest that DON is blocking a system in the embryo which appears or begins to function some hours after fertilization, and is uniquely susceptible to the action of DON. It further appears that the protective agents are acting in a non-competitive manner, replacing an essential substance whose presence or function has been blocked by DON.

The protective action of the purines suggests that DON and azaserine interfere with purine synthesis in the sand-dollar embryo; this has already been demonstrated in bacteria(12,13), tumors, leukemic and normal tissues(14,15) and pigeon liver system(16, 17). In the pigeon liver system, this has been shown to be due to the specific inhibition of one reaction in purine synthesis, the conversion of formylglycinamide ribotide (FGAR) to formylglycinamidine ribotide (FGAM). Glutamine is the natural substrate of this reaction, and the antimetabolites inactivate the enzyme system involved in transferring an amino group to FGAR to form FGAM. Glutamine added before azaserine or DON *in vitro* will protect this enzyme to some extent, but glutamine is of no protective value when added after the antimetabolites(17). There is evidence that higher concentrations of azaserine and DON affect other systems(15,17), presumably by interfering with other reactions involving glutamine. DON is about 20 to 40 times as active by weight as azaserine *in vivo*(2,15) and *in vitro*(17); differences of the same magnitude as those found in the sand-dollar studies. These antimetabolites appear to be considerably more active, however, in inhibiting sand-dollar development. Thus, the amount of DON inhibiting FGAR to FGAM in the pigeon liver system (estimated at 7.5×10^{-5} M)(17) and the concentrations of azaserine inhibiting bacterial growth (6×10^{-6} M)(13) may be contrasted with the concentration of DON blocking the sand-dollar embryo (1.75×10^{-9} M).

While the locus of action of azaserine and DON is well-defined, there is evidence that the effects of DON, or the activity of protective substances are not the same in several biological systems. Thus, for example AIC and adenine protect the chick embryo to some extent against the teratogenic effect of DON, and guanine is ineffective(2), whereas AIC does not protect the sand-dollar embryo, and guanine is more protective than adenine against DON. In contrast to the marked sensitivity of the sand-dollar embryo to azaserine, the frog embryo is reported to be unaffected when exposed to a 0.25% solution of azaserine (25,000,000 milligamma/10 cc) over a 12-day period(18).

Why does the interruption of *de novo* synthesis of purines by DON and azaserine fail to produce a developmental block until the blastula or early gastrula stage, when cell di-

vision is proceeding actively prior to this stage? The fertilized egg contains a large amount of RNA, and relatively little DNA. Hoff-Jorgensen and Zeuthen(19) describe cytoplasmic deoxynucleosides in high concentrations in the sea-urchin and frog egg cytoplasm, which may serve as precursors of DNA. There is good evidence that the DNA does not come from cytoplasmic RNA, but is formed independently(20,21,22). Abrams (22), on the basis of glycine C^{14} studies, states "the larger part of the DNA purines (in sea-urchin embryos) probably were derived from an endogenous precursor the nature of which is unknown at present." He estimates that 75% of DNA guanine and 88% DNA adenine are obtained from endogenous sources, the glycine label being twice as high in the DNA guanine as compared to DNA adenine, whereas the RNA purines are equally labelled. Hultin(23) has observed that the rate of incorporation of C^{14} formate into purines of the sea-urchin embryo was "rather low during the first hours of development, but rapidly increased during the early blastula stage."

If preformed endogenous purine precursors are present in the embryo, the purine deficiency produced by DON might not be manifest until the precursors were exhausted. DON and azaserine, as extraordinarily potent antimetabolites, may prove useful in analyzing the events associated with nucleic acid synthesis and embryonic development.

Summary. 1. DON and azaserine interrupt the development of sand-dollar embryo at mid-blastula and early gastrula stages. The minimum dose of DON producing consistent effects is 3 milligamma/10 cc of sea-water; azaserine is approximately 1/32 as active. Large doses of DON up to 1 mg/10 cc of sea-water have no appreciable effect on fertilization, cleavage or early development. 2. Various physiological purines and derivatives will protect against the action of DON and azaserine, even when added up to 12 hours after fertilization. The most active ones are guanine, hypoxanthine and inosine. The protective action of these substances appears to be non-competitive, and they are not effective against large doses of DON and azaserine. 3. In view of the fact that DON and azaserine, acting as glutamine antagonists, apparently interrupt the *de novo* synthesis of purines, it is suggested that embryonic development in the sand-dollar is blocked at the time when DNA production, initially supplied by endogenous purine precursors, becomes dependent on *de novo* purine synthesis.

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1. Dagg, C. P., Karnofsky, D. A., *J. Exp. Zool.,* 1955, v130, 555.

2. ———, *Fed. Proc.,* 1956, v15, 238.

3. Murphy, M. L., Karnofsky, D. A., *Cancer,* 1956, v9, 955.

4. ———, *Proc. Soc. Clin. Invest.,* 1956, v35, 726.

5. Thiersch, J. B., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 33.

6. Friedman, M. H., *Am. Vet. Med. Assn.,* 1957, v130, 159.

7. Krahl, M. E., *Biol. Bull.,* 1950, v98, 175.

8. Brachet, J., *Chemical Embryology,* Interscience Publishers, N. Y., 1950.

9. Harvey, E. B., *The American Arbacia and other Sea Urchins,* Princeton Univ. Press, Princeton, 1956.

10. Tyler, A., *The Collecting Net,* 1949, v19, 19.

11. Stevens, M. A., McGrath, D. M., Smith, H., Brown, G. B., (in press).

12. Skipper, H. E., Bennett, L. L., Jr., Schabel, F. M., Jr., *Fed. Proc.,* 1954, v13, 298.

13. Tomisek, A. J., Kelly, H. J., Skipper, H. E., *Arch. Biochem. and Biophysics,* 1956, v64, 437.

14. Bennett, L. L., Jr., Schabel, F. M., Jr., Skipper, H. E., *ibid.,* 1956, v64, 423.

15. Moore, E. C., LePage, G. A., *Cancer Research,* 1957, v17, 804.

16. Levenberg, B., Buchanan, J. M., *J. Am. Chem. Soc.,* 1956, v78, 504.

17. Levenberg, B., Melnick, I., Buchanan, J. M., *J. Biol. Chem.,* 1957, v225, 163.

18. Bieber, S., Hitchings, G., *Cancer Research,* 1955, suppl. 3, 80.

19. Hoff-Jorgensen, E., Zeuthen, E., *Nature,* 1952, v169, 245.

20. Schmidt, G., Hecht, L., Tannhauser, S. J., *J. Gen. Physiol.,* 1948, v31, 203.

21. Villee, C. A., Lowens, M., Gordon, M., Leonard, E., Rich, A., *J. Cell. and Comp. Physiol.,* 1949, v33, 93.

22. Abrams, R., *Exp. Cell. Res.,* 1951, v2, 235.

23. Hultin, T., *ibid.,* 1957, v12, 518.

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