Effects of Ethyl Carbamate (Urethan) and Related Compounds on Early Developmental Processes of the Leopard Frog, *Rana pipiens*

DONALD B. McMillan AND HELEN I. BATTLE

(Department of Zoology, University of Western Ontario, London, Ontario, Canada)

Urethan (ethyl carbamate) has been used as a laboratory anesthetic for many years, but only within the past decade have its carcinogenic (9) and carcinoclastic (8) properties been demonstrated. The present investigation was undertaken to further elucidate the mode of action of the urethan molecule by a comparison of its effects with those of closely related compounds using the early developmental processes of the frog as indicators. If any specific component of the molecule is responsible for its characteristic effects on a developing embryo, chemicals with constituents resembling this component should induce corresponding effects; otherwise the action might be ascribed to the molecule as a unit.

MATERIALS AND METHODS

Preliminary experiments were designed to determine the range of concentrations of urethan which would permit development of the eggs and larvae of *Rana pipiens*. Blastulae, mid-gastrulae, and newly hatched larvae (4.0 mm. in length), obtained from a natural spawning, were subjected to a graded series of concentrations (11-140 mM) in pond water. Finger bowls containing approximately 150 eggs or larvae in 200 ml. of the respective concentrations were maintained at room temperature (20° – 24° C.) for 7 days.

In subsequent experiments, eggs were obtained by induced ovulation according to the pituitary injection method of Rugh (11). Initial observations were made upon the time of appearance of various readily observable embryonic stages (i.e., early cleavage and gastrulation) in urethan. Parallel experiments were carried out to determine the effect of certain chemicals with molecular components similar to those of the urethan molecule. One hour after fertilization, groups of approximately 150 eggs were placed in finger bowls containing 200 ml. of the experimental solutions and were maintained at 17° ± 1° C. Observations were made on the time of appearance of the first, second, and third cleavage planes, early gastrulation (the initiation of the dorsal lip of the blastopore), and late gastrulation or Shumway (13) stage 12 (blastopore one-fifth of the diameter of the egg). The eggs in each concentration were examined at intervals of 5–20 minutes during cleavage stages, and at longer intervals during gastrulation, and the percentage attaining a given stage was recorded. For any one series of concentrations, comparisons were made with control eggs from the same spawning.

On the basis of preliminary experiments to determine the effective range of concentrations of each chemical compound, the following series were selected:

- Urethan (NH₂COOC₂H₅), 1.1, 5.6, 11, 22, 34, 45, 56, 84 mM;
- Ammonium thiocyanate (NH₄SCN), 0.56, 1.1, 5.6, 11, 22, 56, 110, 220 mM;
- Ammonium chloride (NH₄Cl), 0.56, 1.1, 2.8, 5.6, 8.4, 11, 22, 56, 84, 110 mM;
- Thiourea (NH₂CSNH₂), 22, 45, 84, 110, 140, 170, 220, 340, 380, 450, 500, 560 mM;
- Urea (NH₂CONH₂), 22, 66, 110, 170, 220, 340, 450, 560 mM;
- Ethanol (C₃H₇CH₂OH), 110, 220, 340, 450, 560 mM.

RESULTS

Preliminary experiments on the susceptibility to urethan during different stages of development.—Blastulae, gastrulae, and newly hatched larvae of *Rana pipiens* subjected to urethan in concentrations from 11 to 140 mM exhibited a progressive retardation of growth and differentiation in proportion to concentration. Retardation was particularly pronounced in the cephalic regions including the gill anlagen. Various anomalies were of frequent occurrence, namely lordosis and extensive coelomic edema, together with great dilatation and stasis of the vitelline plexus. Epithelial hyperplasia was also induced and exhibited a random
distribution on the epidermal surface. In general, the more advanced the embryo on initial subjection, the greater was its resistance to urethan. Development of the early blastula was almost completely arrested in the 56 mM solution, while gastrulae subjected at this concentration developed into microcephalic larvae which failed to hatch. The development over a 7-day period for subjection from the gastrula stage is illustrated in Figure 1. A concentration of 110 mM was necessary to block the growth of the 4-mm. newly hatched larvae, while development was progressively retarded in concentrations from 11 to 84 mM.

Effects of continuous exposure to urethan and other related chemical compounds on early cleavage and gastrulation.—Table 1 is a summary of the effects of urethan and related chemical compounds on the progressive attainment of the first, second, and third cleavages and on the initiation and termination of gastrulation.

In all concentrations of urethan from 5.6 to 56 mM, first and second cleavages were completed with the exception of 11 per cent of the eggs in 56 mM (Chart 1). Concentrations of from 22 mM to 56 mM progressively retarded development. The third cleavage occurred in only 11 per cent of the eggs in the 56 mM concentration but was completed in all lower concentrations, although considerably delayed over the range of from 22 to 45 mM. The initiation of gastrulation was progressively retarded in concentrations of from 11 to 94 mM, and Shumway stage 12 in concentrations of from 5.6 to 22 mM. Thus, it is evident that with continuous subjection there is an increasing susceptibility to urethan from cleavage through gastrulation.

The ammonium compounds (ammonium thiocyanate and ammonium chloride) were less toxic to early cleavage than was the urethan. Ammonium thiocyanate concentrations of 56 mM had no effect on the time of appearance of the first cleavage, but second and third cleavages were progressively retarded from 22 to 56 mM. Concentrations of ammonium chloride of 84 mM did not affect the first cleavage, but for the second and third cleavages the concentrations decreased to 2.8 and 1.1 mM, respectively. Gastrulation was more affected by the ammonium compounds than by equimolar concentrations of urethan. It was initiated in all eggs in concentrations up to 11 mM of both ammonium compounds but was considerably retarded at the upper limit of the concentrations employed. Shumway stage 12 was attained only in the most dilute ammonium thiocyanate solution, namely, 0.56 mM, where 92 per cent of the eggs completed gastrulation. In the ammonium chloride, 20 per cent of the eggs in the 0.56 mM solution, 34 per cent in the 1.1 mM solution, and none in the 2.8 mM solution progressed to this stage.

Thiourea, urea, and ethanol had less effect on cleavage and gastrulation than did equimolar concentrations of urethan, since molar concentrations which permitted development were always in excess of those of urethan. The first cleavage did not occur in concentrations of thiourea greater than 450 mM, while concentrations greater than 320 mM inhibited second and third cleavages. In

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<tr>
<th>DEVELOPMENTAL STAGE</th>
<th>First cleavage</th>
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<th>Third cleavage</th>
<th>Initiation gastrulation</th>
<th>Shumway stage 12</th>
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Chart 1.—The effect of urethan in concentrations from 5.6 to 56 mM on the attainment of cleavage and gastrulation stages in Rana pipiens exposed continuously from 1 hour after fertilization.

Chart 2.—The effect of 22 mM concentrations of various chemicals (ammonium thiocyanate, ammonium chloride, urethan, thiourea, urea, and ethanol) on the attainment of cleavage and gastrulation stages in Rana pipiens exposed continuously from 1 hour after fertilization. This is a composite graph compiled from data obtained in several experiments. The time scale in each instance has been adjusted with a common control. Thiourea, urea, and ethanol at this concentration did not alter the developmental rates with the exception of urea during blastopore closure (5) and are accordingly included with the control. The 22-mM solutions of the ammonium compounds were lethal during gastrulation and therefore do not appear on the graph designating blastopore closure.
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urea, concentrations permitting these three cleavages were 560 mM, 340 mM, and 220 mM, respectively. The process of gastrulation was more susceptible to the action of urea than to that of thiourea. It was initiated in all concentrations of urea up to and including 110 mM, but Shumway stage 12 was attained by only 19 per cent of the eggs in the latter solution. Although greatly delayed, gastrulation was completed in 98 per cent of the eggs in the 220 mM concentration of thiourea. First and second cleavages were completely blocked by 560 mM ethanol and third cleavage by 430 mM. Gastrulation was initiated with little or no delay in 340 mM solutions, but blastopore closure was retarded in this concentration.

In all solutions when gastrulation was successfully completed, development proceeded to hatching, with the exception of eggs in the ammonium compounds. In the latter, although small larvae developed, they failed to free themselves from their gelatinous coats.

Chart 2 illustrates the effects of 22 mM concentrations of the various agents on the completion of the five recorded embryonic stages. This concentration was selected for comparative purposes, since, for urethan, it proved to be the maximal concentration which permitted the completion of Shumway stage 12. In addition, at this concentration, urethan is the only one of these compounds which markedly affects both the first and second cleavages. Third cleavage was delayed by 22 mM ammonium chloride as well as by the equimolar urethan. This concentration of both ammonium compounds had a greater retarding effect than urethan on the initiation of gastrulation, while equimolar concentrations of thiourea, urea, and ethanol had no apparent effect. Urethan, in 22 mM concentration, delayed the completion of gastrulation, while in the corresponding concentration of both ammonium compounds it was entirely blocked. The toxicity of 22 mM urea appeared to increase during gastrulation, and consequently overgrowth of the blastopore was considerably delayed, while neither thiourea nor ethanol had any apparent effect.

DISCUSSION

The effects of urethan described in the preliminary experiments of this paper are of the types recorded by many experimental embryologists for the alteration of developmental processes by a variety of physical and chemical agents. Two effects of more limited occurrence, however, are the induction of coelomic edema and epithelial hyperplasia. Coelomic edema has been induced in the frog embryo by means of x-rays (12), and together with vascular stasis is also recorded for the urethan-treated zebra fish larva (1). Epidermal hyperplasia has been described for frog embryos developed from overripe eggs (14), and by treatment with 3,4-dinitrophenol (5), and for the urethan-subjected zebra fish larva (1). This tissue has the appearance of neoplastic growth, and its induction suggests a relationship to the carcinogenicity of the agent.

There is a single record in the literature (10) of urethan treatment of the frog egg (Rana temporaria). Concentrations above N/30 inhibited cleavage and blastulation, while concentrations from N/100 to N/50 progressively retarded development. In the experiments described here, urethan, in approximately these concentrations, has been shown to exert a similar retardation and inhibition on the embryonic processes of cleavage and gastrulation. The early inhibition of cleavage by urethan corresponds to that recorded for the eggs of various sea urchins (2, 7).

The ammonium compounds (ammonium thiocyanate and ammonium chloride) proved highly toxic to the process of gastrulation but had a lesser effect on cleavage. These compounds ionize readily, whereas the others employed do not. Their toxic nature may thus have been attributable to the free ammonium or other ions. It is evident that urethan exerts a more marked inhibition on cleavage and gastrulation than equimolar concentrations of the nonionizing compounds, thiourea, urea, and ethanol. Corman et al. (4) have demonstrated that 10 times as much ethanol as urethan is required to retard division of Tripneustes eggs and have concluded that the ethyl group cannot be considered as the means by which urethan affects cell division.

Larsen (6) has theorized that urethan in the body might be spontaneously or enzymatically hydrolyzed, forming ethanol, carbon dioxide, and ammonia. Administration of alcohol, sodium bicarbonate, ammonium carbonate, or ammonium chloride in molar concentrations comparable to the optimal oncogenic dose of urethan, however, failed to increase the incidence or multiplicity of lung tumors in strain A mice. In the present investigation, the effects of urethan on cleavage and gastrulation have been demonstrated to be of entirely different degrees from those produced by the ammonium compounds, the ureas, and ethanol. Corman (3) considers that all evidence would seem to indicate that the mechanism of urethan inhibition is due to the adsorption of the intact molecule rather than a chemical combination. He demonstrated that substitution on either or both ends of the urethan molecule augments its
FIG. 1.—The effects of urethan on the development of Rana pipiens subjected continuously from the mid-gastrula stage.
inhibition of Arbacia eggs and hence concluded that the amino or ester linkages appear to be unlikely sites for the narcotic to combine with cellular components. From the present experiments, it would seem improbable that any one component of the urethan molecule is solely responsible for the effects observed, and hence its action would appear to be attributable to the molecule as a whole.

SUMMARY

1. Blastulae, gastrulae, and newly hatched larvae of *Rana pipiens* exposed to urethan concentrations from 11 to 110 mm undergo retardation of growth and differentiation. Early stages are more susceptible than later ones, and the effects are proportional to concentration.

2. No specific types of anomalies can be ascribed to urethan, with the possible exception of coelomic edema, vascular stasis, and epidermal hyperplasia.

3. The effect of urethan on the formation of the first three cleavage planes and on the process of gastrulation is unlike the effect of corresponding concentrations of a variety of other agents (ammonium thiocyanate, ammonium chloride, thiourea, urea, and ethanol), with molecular components resembling certain parts of the urethan molecule. The molar concentration required to induce corresponding degrees of retardation by these several agents is markedly different from that of urethan.

4. It would appear that the effect of urethan is due to the action of the molecule as a whole and not to the agency of any specific component.

REFERENCES

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