

THE ACTION OF UREA ON DIAPAUSE IN EGGS OF *ACHETA COMMODUS* (WALK.) (ORTHOPTERA: GRILLIDAE)

By T. W. HOGAN*

[Manuscript received March 1, 1961]

Summary

Urea, at concentrations of 0.04–0.08M, was found to prevent the onset of diapause in eggs of *Acheta commodus* (Walk.) when applied during the prediapause stages of development.

Higher concentrations, up to 0.24M, were required to terminate diapause in eggs that had already entered diapause. The rate of termination declined as the concentration of urea was reduced; the lowest concentration causing appreciable increase in the rate of termination was about 0.006M. The upper limit to the effective strength was imposed by toxic effects of urea.

An additional experiment is described in which interrelation between the effects of exposure to low temperature and the application of urea on the rate of elimination of diapause was measured.

I. INTRODUCTION

The rate of termination of diapause in eggs of the field cricket *Acheta commodus* (Walk.) has a negative temperature coefficient within the range -16.5°C to $+5^{\circ}\text{C}$ (Hogan 1960b). Similar responses to temperature have been obtained in eggs of *Leptohylemyia coarctata* Fall. (Way 1960).

A negative temperature coefficient for a steric change in a protein, viz. the initial stages of the denaturation of β -lactoglobulin by urea, has been demonstrated by Jacobsen and Christensen (1948).

This was of interest because certain of the characteristics of diapause, including its termination by physical agencies, such as by abrasion, would be most readily explained in terms of a steric change. In view of this and since a negative temperature coefficient for a biological reaction is a rare phenomenon, the possibility that diapause in *Acheta* is terminated by a similar process to the denaturation of β -lactoglobulin was investigated.

In order to incorporate chemicals into the eggs, use was made of the characteristics of orthopteran eggs whereby at one stage of development a considerable quantity of water is absorbed over a relatively short period. In *Acheta* an amount almost equal to the original weight of the egg is absorbed during the fourth and fifth days at 27°C (Browning 1953), commencing almost coincidentally with the onset of diapause (Hogan 1960a).

In subsequent experiments it was found that urea could eliminate diapause, whether applied before or after water uptake, suggesting that the action might be on the egg cuticle. Tests with radioactive isotopes, however, have shown that absorp-

* Plant Research Laboratory, Department of Agriculture, Burnley, Vic.

tion of the urea into the egg takes place. These observations will be reported in a separate paper.

II. METHODS AND MATERIALS

The eggs were obtained from cultures of field crickets maintained in the laboratory. To guard against the selection of strains the cultures were only one generation removed from crickets collected in the field. Conditions for oviposition and for incubation of the eggs were as described in a previous paper (Hogan 1960a).

The main criterion used to measure the effect of treatments on diapause was the percentage of eggs that hatched without evidence of diapause. The median period required by diapause-free eggs to complete their development at 27°C is 15 days (Hogan, unpublished data).^{*} In these experiments eggs that hatched within 3 days of the median period were considered to be diapause-free. However, when the effect of treatment was not very marked the measure used was the period taken for 50% of the eggs to hatch. This is termed the median effective duration of exposure (M.E.D.E.). An alternative measure in such cases was the percentage hatching in successive weeks after the hatching of diapause-free eggs.

The urea solutions were prepared from analytical grade reagents.

In each experiment the eggs were placed on disks of blotting-paper saturated with a solution of the chemical under test and held in sealed plastic tubes at 27°C. Three replicates of 25 eggs per tube were used in each treatment. The control treatment was the same except that distilled water replaced the chemical solution and the percentage of eggs in this treatment that hatched without evidence of delay was taken to be a measure of the strength of diapause in the eggs. Actually this measures the strength of the tendency to enter diapause. These two factors seem to have a positive relationship, but the exact correlation has not been measured. In Section IV the need for a more satisfactory criterion of strength of diapause is indicated.

The term "elimination of diapause" as distinct from "termination of diapause" has been used where the eggs developed, after treatment, without evidence of diapause although they may have experienced one of brief duration.

III. RESULTS

(a) *Effect of Denaturants*

Solutions of urea were applied to prediapause eggs by the method described in Section II. This proved highly toxic at the concentrations normally used for the denaturation of protein (4–7M). This toxicity, however, was assumed to be an indication of absorption of the urea by the eggs and further tests were carried out at less toxic levels. These were in several series, and in the series at the lowest concentrations, in which only slight toxic effects were observed, a definite effect on the elimination of diapause was obtained at 0.08M (Table 1). The experiments were, therefore, continued with urea and are described in the later sections of this paper. Tests were also made on other denaturants. L-Guanidine hydrochloride at concentrations of

^{*} Browning quotes 13 days for the mean duration at 26.8°C but this was after 30 days at 12.8°C during which embryogenesis proceeds slowly.

0.01–2M had no visible effect. Phenylthiourea (0.01M) was highly toxic whilst thiourea at concentrations of 0.01–1M was also highly toxic but with some indication of an effect on diapause in surviving eggs at the lower concentrations.

It was concluded that the lack of toxicity of 2M guanidine probably meant that it was not absorbed into the eggs. Any effects on diapause that phenylthiourea might have had were obscured by its high toxicity at the concentration tested. The rate of elimination of diapause in the eggs surviving concentrations of 0.01–0.04M thiourea was slightly higher than for the control. Further investigation of both of these materials would seem to be worth while.

TABLE 1
PERCENTAGE OF VIABLE EGGS THAT DEVELOPED WITHOUT EVIDENCE OF DIAPAUSE AFTER EXPOSURE, DURING THE PREDIAPAUSE STAGES, TO SOLUTIONS OF UREA AT THE CONCENTRATIONS INDICATED

Urea Concn. (M)	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch	Mortality (%)
0	12.5	4.7	5
0.02	13.2	5.2	0
0.04	16.5	8.0	4
0.08	41.2	43.4	32

* H = percentage hatch; difference for significance at the 5% level = 13.1, at the 1% level = 19.0.

(b) *Optimum Concentration of Urea*

In the experiments described in Section III (a) a marked increase in the percentage of eggs developing without evidence of diapause was obtained with a 0.08M solution of urea.

In order to determine the optimum concentration for this effect urea solutions ranging from 0.01 to 0.16M (see Table 2) were applied to eggs not more than 24 hr old, and kept in contact with them throughout the incubation period at 27°C. The effect of the treatments was measured by the percentage hatching in a period corresponding to diapause-free hatching, and by a further count 2 weeks later.

Although the maximum response is estimated to be at 0.03M (Table 2) it is evident, from a consideration of differences for significance, that the maximum is not sharply defined in the range 0.02–0.04M. When further counts were made after a total period of 31 days the percentage hatches at 0.02, 0.03, and 0.04M were closely similar (59.6, 64.4, and 65.7), and substantially higher than the percentage hatch at 0.08M (18).

A series of low concentrations was tested separately (Table 2) and, when measured in terms of the period of exposure required for a 50% hatch, demonstrated that, for these eggs, the threshold was about 0.006M; and that the rate of elimination increased with increase in the concentration of urea.

(c) *Timing and Dosage of Urea*

In the previous experiments the eggs were treated with the urea solutions shortly after oviposition and remained there until the completion of hatching. The assumption was made that the urea was incorporated into the eggs during the process of water uptake, but this had not been proved.

TABLE 2
EFFECT OF DIFFERENT CONCENTRATIONS OF UREA ON THE RATE OF ELIMINATION OF DIAPAUSE
WHEN INCUBATED AT 27°C

Rate Measured by Diapause-free Hatching				Rate Measured by Period Taken for 50% Hatch	
Urea Concn. (M)	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch after 17 Days	Mortality (%)	Urea Concn. (M)	Median Effective Duration of Exposure (days)
0	13.7	5.6	3	0	54
0.004	20.4	12.2	3	0.006	47
0.02	27.7	21.6	7	0.01	40
0.03	39.6	40.6	3	0.02	35
0.04	25.3	18.3	0	0.04	23
0.08	19.9	11.6	4		
0.16	16.1	7.7	36		

* H = percentage hatch; difference for significance at the 5% level = 21.1, at the 1% level = 29.4.

In order to determine at what stage of development the action took place, the contact of the eggs with the urea solution was restricted to particular stages of development.

(i) *Effect of Exposure to Urea during the Period of Water Uptake.*—These effects were compared with those of exposure to urea during the whole of prediapause development. The results (Table 3) show that the longer the period of exposure during prediapause development the more marked is the effect. This suggests that the total period of exposure is important rather than just the period of water uptake. The lower percentage hatching of the eggs in contact with urea throughout the incubation period possibly resulted from some overdosing, sufficiently unfavourable to reduce the rate of development. It was not because of lethal effects.

TABLE 3
EFFECTS OF UREA WHEN APPLIED AT DIFFERENT STAGES OF DEVELOPMENT OF THE EGG

Urea (0.03M) Applied during Prediapauses Stages				Urea (0.04-0.24M) Applied during Diapause				
Day of Treatment	Arcsin (H°)* (degrees)	Retransformed Percentage Hatch	Mortality (%)	Urea Concn. (M)	Arcsin (H°)+ (degrees)	Retransformed Percentage Hatch after 17 Days	Mortality (%)	Hatch+ after 30 Days (%)
No urea	14.4	6.2	0	0	5.5	1.0	1	11
1st-5th	26.6	20.0	0	0.04	11.8	4.2	4	43
1st-6th	43.8	47.9	0	0.08	31.2	26.8	8	87
4th and 5th	21.5	13.4	3	0.16	61.2	76.8	12	86
4th, 5th, and 6th	26.4	19.8	0	0.24	48.6	56.3	53	47
Continuous	21.9	13.9	3					

* H = percentage hatch; difference for significance at the 5% level = 12.7, at the 1% level = 17.3.

† Difference for significance at the 5% level = 28.1, at the 1% level = 39.9.

‡ Values not transformed.

(ii) *Effect of Exposure to Urea after Water Uptake.*—As the result of evidence obtained in several pilot tests, an experiment was set up to determine the effects of the application of urea after water uptake had been completed and the eggs had entered diapause. For this purpose the eggs were held at 23°C for 14 days before treatment, and higher concentrations of urea, from 0.04 to 0.24M, were used. The results (Table 3) reveal that these concentrations of urea could cause the termination of diapause. The most effective concentration in this case was 0.16; beyond this, at 0.24M, the mortality was high (53%).

TABLE 4
INFLUENCE OF UREA ON THE RATE OF ELIMINATION OF DIAPAUSE IN EGGS PREVIOUSLY EXPOSED TO LOW TEMPERATURE

Urea Concn. (M)	No. of Days at 12°C	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch	Urea Concn. (M)	No. of Days at 12°C	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch
0.04	0	38.9	39.4	0	0	23.7	16.2
0.04	5	60.0	75.0	0	5	41.1	43.2
0.04	14	24.1	16.7	0	14	57.0	70.3

* H = percentage hatch; difference for significance at the 5% level = 23.6, at the 1% level = 33.4.

Browning and Forrest (1960) have shown that in non-diapause eggs exchange of water occurs at all stages of development, but this would not be expected to apply to diapause eggs. It might be supposed that the urea was acting only on the cuticular coverings causing a change in their permeability, but an investigation with radioactive isotopes has shown that the urea is, in fact, absorbed into diapause eggs.

(d) *Combined Effect of Low Temperature and Urea*

Since urea can cause the elimination, or termination, of diapause in *Acheta*, the possibility that it is the agent under natural conditions must be considered. It is true that the usual end-product of nitrogen metabolism in insects is uric acid, but the presence of urea has been recorded in a number of insect species (see, for example, Bheemeswar 1958). Hence the synthesis of urea, and its accumulation until an effective concentration is reached, could be the mechanism by which diapause in *Acheta* is eliminated when eggs are exposed to temperatures of about 10°C.

If urea is indeed synthesized at low temperatures then the effectiveness of applied urea should increase after the eggs have been exposed to such, provided the optimum dosage of urea is not exceeded. Beyond this latter level unfavourable effects would be expected to occur, corresponding to the responses obtained when concentrations of urea beyond the optimum are applied to the eggs.

An experiment was therefore carried out in which eggs, not more than 16 hr old, were held at 12°C for 0, 5, and 14 days and then treated with 0.04M urea during

incubation at 27°C. Another group, from the same batch of eggs, was given the same treatments but without the addition of urea.

Table 4 shows that after an exposure of 5 days at 12°C. followed by treatment with 0.04M urea, the rate of elimination of diapause was higher than after either of these treatments alone, but after 14 days at low temperature the rate was substantially lower.

If it be assumed that the optimum concentration for these eggs was higher than 0.04M, then the data are consistent with a hypothesis that urea is synthesized during exposure to low temperature; but the results do not exclude other interpretations.

IV. DISCUSSION

The foregoing experiments have demonstrated that urea, a well-known denaturant, has the capacity to terminate diapause from eggs of *A. commodus*. The feasibility of the action being denaturant is supported by some of the characteristics of diapause in other species, e.g. the response to physical agencies, the rapidity of such responses, and the reversibility of diapause recorded by Salt (1947). The fact that a negative temperature coefficient has been found for a denaturant action and also exists for the termination of diapause in *Acheta* seems significant.

Against this the chief objection appears to be that the negative temperature coefficient for denaturation in urea, "seems to be rather special to β -lactoglobulin and not to be a general property of the proteins" (Dr. M. Ottesen, personal communication). Another objection is the low concentration of urea found to be effective. However, denaturation, although an extremely complex process, includes comparatively simple steric changes such as the breaking of hydrogen bonds. Klotz (1958), who has reviewed one such change, viz. the hydration of protein molecules, states that the breakdown of the water lattice by urea reveals itself (among other ways) by unmasking of previously inactive groups. As far as unmasking is concerned, "even a 1M solution produces a visible effect" (Klotz, personal communication). Jensen (1955) remarks that relatively mild conditions may affect sulphhydryl-disulphide interchange—again affecting the reactivity of proteins. Moreover, although concentrations of urea as low as those used in the experiments on *Acheta* have not been recorded as causing steric changes, the long periods of exposure at low concentrations may have effects not observable with *in vitro* chemical reactions.

Urea is effective in increasing the rate of termination of diapause in *Acheta* when applied at concentrations within the range 0.006–0.16M. (The concentration at the site of action is not known.) The upper limit of this range is set by toxic effects, the onset of which overlaps those concentrations most effective in terminating diapause. This is reminiscent of the effect of subzero temperatures (Hogan 1960b).

The optimum concentration of urea, measured by diapause-free hatching was 0.08M in one test (see Table 1) and 0.03M in another (Table 2). This discrepancy is not due to a difference in the onset of mortality which was the same in both experiments. Nor does it appear to be linked with the intensity of diapause as measured by the control treatments, as this, too, was very similar. A number of possibilities exist but one question that arises is whether the method of measuring the intensity

of diapause is a satisfactory one. Further experiments will be necessary to elucidate this point.

Apart from the nature of the action of urea there is the question as to whether urea could be the agent causing the termination of diapause under natural conditions. The effectiveness of exposure to temperature of about 10°C for 1–3 months could be readily explained in terms of the slow synthesis of urea during this period and its accumulation until an effective concentration is reached. Perhaps at higher temperatures a balance between breakdown rate and synthesis would account for the decrease in effect. Schneiderman (1956) has suggested that such a process (in relation to an unknown agent) could be the mechanism of termination in postembryonic diapause.

V. ACKNOWLEDGMENTS

The author wishes to thank Miss J. Kenyon for technical assistance in the conduct of these experiments, Mr. R. Jardine for carrying out the statistical analyses, and Dr. F. H. Drummond for critical discussion of the manuscript.

VI. REFERENCES

- BHEEMESWAR, B. (1958).—Some aspects of amino acid metabolism in insects. In "Symposium XII: Biochemistry of Insects". (Ed. L. Levenbook.) pp. 78–89. (Proc. 4th Int. Congr. Biochem., Vienna.) (Pergamon Press: London.)
- BROWNING, T. O. (1953).—The influence of temperature and moisture on the uptake and loss of water in the eggs of *Gryllulus commodus* Walker (Orthoptera, Gryllidae). *J. Exp. Biol.* **30**: 104–115.
- BROWNING, T. O., and FORREST, W. W. (1960). The permeability of the shell of the egg of *Acheta commodus* Walker (Orthoptera, Gryllidae) *J. Exp. Biol.* **37**: 213–17.
- HOGAN, T. W. (1960a).—The onset and duration of diapause in eggs of *Acheta commodus* (Walk.) (Orthoptera). *Aust. J. Biol. Sci.* **13**: 14–29.
- HOGAN, T. W. (1960b).—The effects of sub-zero temperatures on the embryonic diapause of *Acheta commodus* (Walk.) (Orthoptera). *Aust. J. Biol. Sci.* **13**: 527–40.
- JACOBSEN, C. F., and CHRISTENSEN, L. K. (1948).—Influence of temperature on the urea denaturation of beta-lactoglobulin. *Nature* **16**: 30–1.
- JENSEN, E. V. (1959).—Sulphydryl-disulfide interchange. *Science* **130**: 1319–23.
- KLOTZ, I. M. (1958).—Protein hydration and behaviour. *Science* **128**: 815–22.
- SALT, R. W. (1947).—Some effects of temperature on the production and elimination of diapause in the wheat stem sawfly *Cephus cinctus* Nort. *Canad. J. Res. D* **25**: 66–86.
- SCHNEIDERMAN, H. A. (1956).—Onset and termination of insect diapause. In "Physiological Triggers". pp. 46–59.
- WAY, M. J. (1960).—The effects of freezing temperatures on the developing egg of *Leptohylemyia coarctata* Fall. (Diptera: Muscidae) with special reference to diapause development. *J. Insect Physiol.* **4**: 92–101.