Phosphorylation with Inorganic Phosphates at Moderate Temperatures

Abstract. *Uridine phosphates may be obtained by heating uridine with inorganic phosphates for 9 months at temperatures as low as 65°C. Under similar conditions, uridine-5′-phosphate, in addition to forming uridine diphosphates, undergoes some dephosphorylation to give uridine.*

A number of publications (*1–4*) have dealt with the formation of nucleotides from nucleosides and inorganic phosphate by dry heating, but with one exception (*1*): they refer to experiments carried out at 160°C. Here we report quantitative results which show that these and related reactions proceed effectively, although more slowly at temperatures as low as 65°C.

Uridine was heated with inorganic phosphates in sealed ampules for 9 months at 65°C. Uridine monophosphates, together with small amounts of uridine diphosphates, were obtained with $Ca(H_2PO_4)_2$ (Table 1). No phosphorylated products (< 1 percent) were detected with $(NH_4)H_2PO_4$, $(NH_4)_2HPO_4$, NaH_2PO_4, Na_2HPO_4, or $CaHPO_4$. In similar experiments in unsealed ampules at 85°C for 6 months, a good yield of uridine monophosphate was obtained with $Ca(H_2PO_4)_2$, and a lower yield was obtained with $(NH_4)_2HPO_4$. However, NaH_2PO_4, Na_2HPO_4, KH_2PO_4, $Ca(NH_4)PO_4$, and $Mg(NH_4)PO_4$ gave negative results.

In an independent series of experiments, uridine-5′-phosphate was heated with inorganic phosphates in unsealed ampules for 6 months at 65°C. Under these conditions, dephosphorylation occurred; the yield of uridine was in the range of 10 to 20 percent, with Na_2HPO_4, KH_2PO_4, $CaHPO_4$, $(NH_4)_2HPO_4$, and $Mg(NH_4)PO_4$, and as high as 50 percent with NaH_2PO_4. Simultaneously, some diphosphates, presumably 2′,5′- and 3′,5′-, were formed. The best yield was obtained with $Ca(H_2PO_4)_2$, and smaller yields were obtained with $(NH_4)_2HPO_4$ and NaH_2PO_4 (Table 2); results with $Mg(NH_4)PO_4$, Na_2HPO_4, and KH_2PO_4 were negative.

In that $Ca(H_2PO_4)_2$ is precipitated only from acid solutions, it seems unlikely that it was ever a common mineral. However, if the oceans ever contained substantial amounts of ammonia and little calcium or magnesium, it is possible that some $(NH_4)_2HPO_4$ was formed by evaporation of shallow pools. When heated, this phosphate could have converted nucleosides to nucleotides. In this respect, $(NH_4)_2HPO_4$ is unique, for, although it is formed from neutral or slightly alkaline solutions, it loses ammonia on heating and then provides an acid environment.

If the hydrolysis of uridine-5′-phosphate to uridine that we observed is a general reaction of phosphate esters, it is unlikely that polymeric materials could ever be obtained under our reaction conditions. Perhaps polymers could be formed at higher temperatures or lower humidities.

Reaction mixtures were prepared by dissolving ^{14}C-labeled uridine or uridine-5′-phosphate in water, dissolving or suspending the inorganic phosphate, and lyophilizing the resulting solution or suspension.

The products were analyzed by paper chromatography in a mixture of isopropyl alcohol, concentrated ammonia, and water (70 : 10 : 20) and in a mixture of 95 percent ethyl alcohol and 1*M* ammonium acetate (7 : 3), *p*H 7.5. To confirm the formation of uridine diphosphates and diuridine phosphate, the products were eluted from the paper and subjected to paper electrophoresis (0.03*M* potassium phosphate buffer, *p*H 7.1, 4000 volts). Uridine monophosphates were eluted and chromatographed again in the isopropyl alcohol, concentrated ammonia, 0.1*M* boric acid system (70 : 10 : 20) to separate uridine-5′-phosphate from uridine-2′(3′)-phosphate.

The products on the chromatograms were located and estimated roughly by viewing under an ultraviolet light and using a radiochromatogram scanner with integrator. For quantitative analyses, the chromatograms were cut into segments, and the radioactivity was determined in a scintillation counter. The yields were calculated as the percentage of total ^{14}C on the paper.

Table 1. Products of reaction mixtures of uridine and inorganic phosphate. pU, uridine-5′-phosphate; Up, uridine-2′(3′)-phosphate; pUp, uridine diphosphate; and UpU, diuridine phosphate.

Temperature (°C)	Inorganic phosphate	Ratio of uridine to phosphate	Uracil	pU	Up	pUp	UpU
65	$Ca(H_2PO_4)_2$	1:1		2.3	0.5		
65	$Ca(H_2PO_4)_2$	1:5		11.3	3.3		1.4
85	$Ca(H_2PO_4)_2$	1:20	7.45	28.3	7.6	14.1	
85	$(NH_4)_2HPO_4$	1:20		2.9*			

* Up and pU were not separated.

Table 2. Products of reaction mixtures of uridine-5′-phosphate (pU) and inorganic phosphate (P_i) at 65°C. pUp, uridine diphosphate.

P_i	pU/P_i	Uridine	pUp
$Ca(H_2PO_4)_2$	1:20	14.7	12.1
$(NH_4)_2HPO_4$	1:20	13.5	4.1
NaH_2PO_4	1:20	45.5	3.4

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Motility of the Turtle Embryo, Chelydra serpentina (Linné)

Abstract. *Periodic motility of turtle embryos was observed during their incubation periods (60 ± 5 days). Cyclic activity was first observed between days 10 to 14; it increased to a peak level of 50 percent of the standard observation period on day 30 ± 5, then declined to low levels until hatching activities were initiated. During the first third of the incubation period, motility of the turtles closely resembled that previously described for chick embryos at similar stages of development.*

Motility of embryonic turtles removed from the nests of *Terrapene carolina* and *Caretta caretta* has been described by Tuge (*1*) and by Smith and Daniel (*2*). They reported spontaneous, nonreflexogenic activity of young embryos, but they were principally concerned with observations of reflex behavior following mechanical stimulation. My investigation of embryonic motility of the snapping turtle combined the methods for harvesting turtle eggs *ex utero* (*3*) with those developed for observing spontaneous periodic motility of the

embryonic chick (*4*). Use of these techniques permitted accurate observations of turtle development under controlled conditions of the laboratory.

Fertile eggs were removed from the uteri of decapitated gravid snapping turtles obtained from a professional collector (*5*). The number of eggs in a single clutch varied from 17 to 52, and, in general, the size of the clutch was related to the size of the turtle. The embryos were located through the shell by candling. Circular openings approximately 1.5 cm in diameter were made above the developing embryos. The eggs of a single clutch were found to be in late gastrula stage of development, and they continued to develop synchronously when incubated under similar conditions of temperature (30°C) and humidity. In lieu of a staged series (*6*), the chronological age in days of incubation was used to designate the stage of development.

The motility of turtle embryos was cyclic, the periods of activity alternating with those of inactivity. Activity of turtle embryos through 45 days of incubation was recorded mechanically on a Sanborn polygraph for a standard 15-minute observation period. Altogether, 27 embryos from five different clutches were used, and 117 recordings of embryonic motility were made throughout the period of incubation (60 ± 5 days). The mean percentages of activity (Fig. 1) and the durations of the phases of activity and inactivity were determined, as they were in similar studies of motility in chick embryos (*7*). The activity of a single embryo was recorded at several different stages of development. Since opening of the shell often caused precocious hatching, the increased motility observed in hatching turtles is not included in this paper.

Spontaneous (nonstimulated) cyclic activity was observed as early as the 10th day of incubation and was obvious in all embryos by day 14. At the time motility was initiated, the embryo had assumed a prone position over the yolk, with its head and neck flexed. The turtle embryo does not undergo torsion but usually comes to rest on its left side, apparently as a result of the development of the allantois and of the force of gravity. By manipulation of the eggs at this stage, some embryos were made to lie on the right side, and their subsequent development appeared normal. The first movements of the embryo originated as a slight "twitch" in the cervical region; this movement flexed the head laterally. The

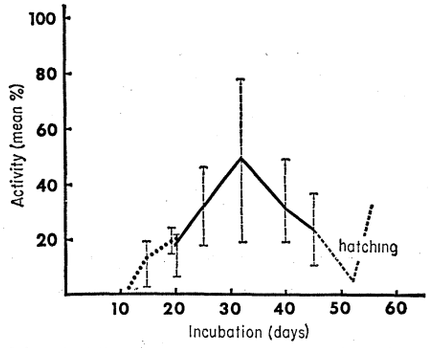

Fig. 1. The mean percentages of activity of the turtle embryos during the incubation period. The dotted line represents the percentages of total body activity. The solid line indicates the percentages of activity of the fore limb, which are equivalent to those of total body activity at these stages. The broken line suggests the activity of the embryos during hatching. Vertical broken lines show the range of activity for individual embryos.

appearance of motility in the cervical region was coincident with an accumulation of pigment along the anteriodorsal surface of the retina. Within a few hours the motions of the head became more pronounced; the head flexed from side to side and incorporated more caudal trunk musculature, in this manner producing the S-waves observed in amphibians (*8*). Also during this period of development, the head was elevated and depressed in a movement similar to nodding, thus forming C-waves (*2*). These C-waves were distinctly different from, and should not be confused with, the bobbing motion of the head which occurred when the head was moved passively by pulsations of the heart. The C- and S-waves which extended caudad and involved the tail, provided most of the body activity observed through the first 18 days of incubation. Mechanical stimuli applied to different regions of the body during this period of development failed to evoke a response.

Activity of the appendages was noted as early as day 16 and was observed in all embryos by day 19. Slight elevations of the forelimbs were first observed during phases of total body activity. At this stage pigment granules were noted aggregating in the skin in the region of the pectoral girdle. Activity of the hindlimbs appeared about 4 to 10 hours later. Stimulation of the appendages during phases of inactivity failed to evoke a response at this time. This early activity of the appendages involved the entire limb; the first movements of a appendicular joint appeared at the developing carpal joint, resulting in an extension and flexion of its paddle-shaped distal end.

By day 22, activity of the fore or hind limbs was equivalent to that recorded for total body activity (Fig. 1). Appendages were independently active at this stage; however, most of the activity of the limbs occurred during phases of total body activity. Evoked response to mechanical stimuli to the fore limbs was obtained from day 22 on, an indication that neuroneuronal and neuromuscular synaptic junctions subserving reflexes were functionally connected at this stage of development. The carapace and plastron were clearly identifiable at this stage, and these imposed serious restrictions on activity of the trunk region. Activity of the head, neck, and tail, as well as of the appendages, appeared random and uncoordinated. These apparent random movements continued through at least 45 days of incubation.

Of considerable interest was the observation that the rhythmic activity of the amnion, which is characteristic of chick embryos, was not observed during the development of the turtle. This supports the findings that in the chick, the cyclic aspects of embryonic motility are unrelated to contractions of the amnion (*9*).

The peak of random motility occurred approximately halfway through the incubation period (30 ± 5 days) (Fig. 1). Afterward, activity declined through day 45, when the embryos became very sluggish. Just before hatching, between 50 and 60 days, embryonic motility increased noticeably.

My observations of the hatching behavior of the turtle were made more difficult by the artificial opening through the shell. In most cases the animals emerged through this aperture prematurely. In these premature hatchlings the yolk sac was still visible in the center of the large umbilical ring. During the final 3 or 4 days of incubation the turtles were extremely sensitive to stimuli; they were capable of following a lure and showed snapping reflexes. Prematurely hatched turtles, as well as normally hatched specimens, were able to perform righting reflexes as soon as they emerged. Normal hatchlings emerged when the calcareous material of the shell became fragile and flaked off, leaving only the soft pliable membranes to be penetrated.

The motility patterns of *Chelydra* embryos and those of chick embryos resemble each other closely in the following ways. Motility is first observed

in the cervical region and spreads caudally, involving eventually all parts of the body. Movements of the limbs are performed at first only in conjunction with the body; they become independent a few days after the inception of motility. Motility is periodic in both forms; activity phases alternate with inactivity phases. Embryos become sensitive to exteroceptive stimulation a few days after the onset of motility, a demonstration of the nonreflexogenic nature of early motility. In both forms, movements are apparently random; the right or left, fore or hind limbs move without evidence of coordination. In brief, this pattern seems to be characteristic of these two amniote groups, reptiles and birds.

The main difference between chick and turtle embryos is in the profile of percentages of activity during incubation. The chick reaches high levels of activity, 80 percent by day 13, which are maintained until just before hatching. In the turtle maximum activity is reached about halfway through the incubation period and has a mean value of only 50 percent; it is not sustained. Periodic activity declines from this point until shortly before hatching.

The attenuated incubation period of the turtle, coupled with its low levels of total activity, make it an excellent animal for use in studies of the qualitative as well as the quantitative aspects of embryonic behavior.

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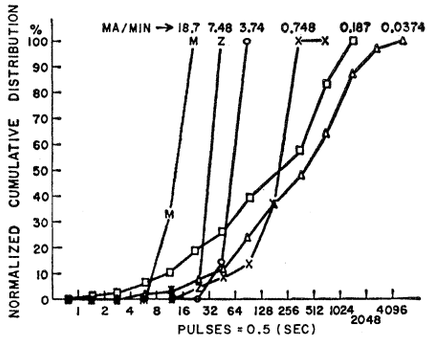

Fig. 1. Normalized cumulative distrbutions for subject 319. The distributions represent the percentage of total responses made at or before each interval of pulses on the abscissa. Different slots are for different rates of increase. Due to the range of interresponse times at different rates of increase of shock, the distributions are based on geometrically increasing intervals of pulses. The abscissa values are given in terms of pulses of shock. Since these occurred at the constant rate of two per second, the abscissa values can be converted to seconds if they are divided by two.

Training and Maintenance of Keypecking in the Pigeon by Negative Reinforcement

Abstract. *Pigeons were trained to peck a key to escape a pulsing shock of linearly increasing intensity. As the rate of increase was varied from 0.0374 milliamperes per minute to 37.4 milliamperes per minute, the intensity at which most pecking occurred varied from 2.2 to 5.0 milliamperes.*

Although pigeons are routinely trained to peck a key for food, they have not been so easily trained to peck a key to escape or avoid electric shock. The difficulty seems to be due, at least partly, to variations in the pigeons' sensitivity to shock. In previous studies with a simple response such as head-lifting to escape and avoid shock, the shock intensity had to be adjusted daily in order to maintain responding (*1*). With key-pecking it was necessary to continually adjust shock intensity, even from moment to moment (*2*).

Yerkes and Dodson (*3*), using rats, found that as a task becomes more complex a given criterion of learning can be attained only with a progressively narrower band of shock intensities. Intensities below this band presumably do not sufficiently motivate the response while intensities above the band elicit responses, emotional or otherwise, that interfere with the response being learned. Perhaps the band of intensities which could support key-pecking in the pigeon is too narrow for informal adjusting procedures to keep the intensity of shock within it. The present experiment was designed to permit automatic adjustment to the effective band of shock intensities. Trains of brief shocks of gradually increasing intensity were presented. Since the tensity increased gradually, several shocks were presented within any band of reasonable width. If fluctuating sensitivity to shock were the main source of difficulty in shaping escape responding in the pigeon with manual adjustment of intensity, such responding should be easily shaped and maintained by this procedure, and the birds' responding should reveal the range and stability of the lower limit of the effective band.

Four pigeons, allowed free feeding in their home cages, were run once a day in a chamber containing an overhead light and a response-key. Shock pulses (35 msec long) were delivered at a rate of two per second through gold wires imbedded under the pigeon's pubis bones. The shocks increased linearly in intensity from 0 to 8.5 ma. Pecks on the key, while the shock was increasing or after it had reached maximum, reset the shock to zero and turned off the overhead light for 3 seconds. Then the light came back on and shock began to increase again.

Shock was 400-v alternating current passed through a fixed resistance of 25 kohms and a motor-driven variac. A gear-changer and a clutch were arranged so that the shock intensity increased from 0 to 8.5 ma at a constant rate (set by means of the gear-changer).

One subject (436) was trained previously to peck a key for food. Two other subjects (270 and 319) were trained to peck a key for food, and then the response was extinguished. They did not peck the key when first tested with the present procedure. Key-pecking was established by setting the rate of increase of shock at 20 ma/min, increasing the maximum intensity from 8.5 ma to 15 ma, and reinforcing successive approximations to key-pecking by reducing the shock suddenly to zero for about 5 seconds. A fourth subject (499) had not been in any previous experiments. Key-pecking for escape was