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Puromycin: Two Distinct Behavioral Effects with Different Temporal Parameters in the Pigeon

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STETTNER, L. J., R A BARRACO, B J. KLAUENBERG AND H NORMILE *Puromycin Two distinct behavioral effects with different temporal parameters in the pigeon* PHARMAC BIOCHEM BEHAV 10(4) 521–525, 1979 — Pigeons were injected intracerebrally with either puromycin (PM) or control saline solution following training for one 12-min session on a visual discrimination Injections were made either immediately following training, 1 hr later or 24 hr later. Retention testing 3 days after training showed that PM produced marked amnesia in the first two groups, but had no effect in the 24 hr condition. However, *all* PM groups were retarded subsequently in the number of days required to reach a 90% discrimination criterion This differentiation of two separate behavioral effects with different temporal gradients suggests that PM may be working through two distinct physiological mechanisms.

Memory Protein synthesis inhibition Puromycin Amnesia Learning deficit Temporal gradient Visual discrimination Pigeon

TEMPORAL variation in effectiveness of antibiotic treatments is interrelated in an important manner with the interpretation (both psychological and physiological) of their effects on memory processes. In considering the effects of antibiotic treatments upon behavior, it must be kept in mind that, even when injections occur immediately after training, there is some period of uninhibited cerebral protein synthesis following the training experience. Following intracerebral injection, the inhibition of cerebral protein synthesis produced by puromycin (PM) in pigeons reaches a maximum of 90% inhibition at 2.5 hours and dissipates significantly by 12 hours. Cycloheximide (CXM) produces a more rapid onset of inhibition of cerebral protein synthesis, reaching over 95% within 45 minutes, maintaining that level for at least 4 hours, and dissipating by 12 hours [21]. These findings are generally consistent with the inhibition data for rodents [10] and goldfish [5].

Previous studies indicate that the behavioral effects of CXM (and the other frequently used glutarimide, acetoxycyloheximide (AXM)) are generally correlated with the temporal parameters of protein synthesis inhibition. Thus, CXM and AXM are usually effective as amnesic agents when injected five minutes to five hours before training [2, 3, 6, 14], marginally effective or ineffective when injected immediately after training [3, 11, 17], and completely ineffective when injected one hour or more after training [3, 5, 15, 20, 22]. Further, the duration of cerebral protein synthesis inhibition produced by anisomycin has also been found to be highly correlated with the magnitude of its amnesic effects [14]. Data from our laboratory are also consistent with these observations since CXM injected immediately after one session of visual discrimination training in pigeons produces a partial amnesic effect [21], while it is ineffective as an amnesic agent when injected immediately following swim escape training [4]

However, the temporal parameters of protein synthesis inhibition for PM-induced retention deficits appear to be quite different. Agranoff and his co-workers, working with avoidance learning in goldfish, report a temporal gradient such that PM injected after training is maximally effective in producing amnesia when treatment is immediate and has no effect when delayed for one hour or more [1,19]. PM also had no apparent effect on retention in 30-day-old rats when injections were delayed 24 hr following training in a swim escape task [4]. On the other hand, the Flexners and their colleagues [7, 8, 18] have repeatedly found that PM produces amnesia for discriminated shock-avoidance with rodents in a Y-maze when injected 24 hours after training. Our investigations with pigeons also suggest a lack of correlation between the overall pattern of inhibition of cerebral protein synthesis and the effects of PM injections on behavior. Thus, in pigeons, as in Japanese Quail [16], PM has a marked amnesic effect on appetitively rewarded visual discrimination learning when injected immediately after training [21]. The PM effect is much larger than that for CXM, despite the fact that the protein synthesis inhibition is more rapid and severe after CXM administration (see above) Further, PM produces an effect on subsequent acquisition training (which occurs sev-

eral days later) *over and above* its amnesic effect for the training just prior to injection, whereas there is no tendency whatsoever for CXM to produce such an effect [21]

Consequently, we conducted a series of studies to evaluate the temporal gradients of the PM effects described above The critical issues are whether (a) the effects of PM on retention match the steep gradient found with CXM (and with PM goldfish), or (b) are they in agreement with the data indicating an effective range of 24 hours or more after training as found in rodents? The former would be broadly consistent with a protein-synthesis-inhibition explanation of the effect, while the latter would be suggestive of the involvement of other mechanisms Further, from a psychological point of view, a steep retroactive gradient would least be consistent with a consolidation blockage hypothesis of PM's effects, whereas the effectiveness of treatments 24 hours after training is usually interpreted as involving a deficit in retrieval mechanisms (Although we cannot, of course, preclude a retrieval-type explanation of immediate effects in the former case nor exclude the possibility of slow acting consolidations effects in the latter)

METHOD

Animals

Fifty-seven male pigeons of the Giant White Carneaux strain, 1–4 years old at the time of training were used. They were housed individually from the time they entered the laboratory until the end of the experiment. Following an initial period of adaptation and ad lib feeding (3 weeks), the pigeons were fed a limited amount of food (Purina pigeon chow) so as to be maintained at 85% of their ad lib body weight throughout the course of the experiment

Apparatus

Training was conducted in a Lehigh Valley Operant chamber, with a 25×37×58 cm high interior equipped with a fan and with 3 transparent pecking keys of 2.54 cm diameter mounted in a horizontal line on one wall 25 cm above the floor of the chamber A stimulus projector was mounted behind each key A rectangular aperture of 5×6 4 cm was centered in the same wall and allowed access to grain through a funnel-like food hopper when a solenoid-operated food tray was activated A 6 W incandescent bulb mounted at the top of the hooper was lit whenever the tray made food available A one-way observation window, 19×21 5 cm, was set into one of the walls perpendicular to the response key wall. Operation of the chamber was controlled automatically and responses recorded through combined solid-state and electro-mechanical circuitry

Procedure

Pretraining When the pigeon had reached 85% of its ad lib weight, it was placed in the test chamber and allowed to eat from the food hopper which was maintained in the operated position continuously until the animal ate readily from it The food tray was then activated intermittently so that the pigeons were trained to approach the food hopper and eat for a short period of time whenever the tray was operated, eating time being gradually shortened until each period of access was limited to 4 sec Key-peck shaping was the next stage, and was accomplished manually by operating the hopper contingent upon successive approximations to pecking at the center key which was lit continuously by a white circle on the projector mounted behind the key. (The side keys were dark and inoperative throughout the entire course of the experiment.)

Once the animal pecked the key with sufficient force to operate the micro-switch attached to it, each peck was reinforced by providing 4 sec of access to food. Pigeons were placed in the apparatus for 3 daily sessions of continuous reinforcement which lasted until they made 80 pecks, during which time the center key was lit continuously except for 15 sec time-out periods which occurred at 1 min intervals Reinforcement was available only when the key was lit. In the next training phase a variable interval schedule (VI) which made food available only once every 25 sec on the average was programmed through use of a tape-loop, and the animals were run for 4 days of VI training to white light, 12 min per day At this point, a persistent pecking response to the white light had been established

Grid training Extensive pilot work had indicated that the animals trained on white light often showed some initial inhibition toward pecking the key when line stimuli were projected upon it during the first discrimination training session (see below) We believed it important to reduce this variability and ensure a prompt and consistent pecking response during this initial brief training period prior to injection Thus, a phase of training was introduced in which both the horizontal and vertical lines (each consisting of 3 black lines, 2 mm wide and 2 5 cm long with a 4 mm white strip between them) were simultaneously projected thus producing a grid stimulus. Pecks at the grid were reinforced on the VI schedule, with the same 1 min on, 15 sec off sequence that had been used in white light training This procedure provided exposure to the line stimuli (in compound form) prior to discrimination thus facilitating immediate pecking during the initial discrimination session, but did not provide any differential reinforcement VI grid training was continued for 5 days, 15 min per day We considered the fact that exposure to the grid stimuli during this period could result in some differential response strength to the stimuli (despite the absence of any differential reinforcement) through selective attention mechanisms by which one aspect of the compound stimulus acquired greater stimulus control of pecking than others. However, since pilot work indicated no obvious tendency for this to occur and since the positive and negative stimuli were counterbalanced within each group during discrimination training (thus prohibiting any systematic error from being introduced due to stimulus bias however generated), it was decided that the advantage of grid training outweighed possible disadvantages

Discrimination training Discrimination training was initiated on the day following the completion of grid training Each discrimination session consisted of 6 presentations of the horizontal lines and six of the vertical lines projected independently upon the response key for 1 min periods, with a 15 sec time out (during which the key was dark but the house light remained on) between stimulus presentations During the presentation of the stimulus designated as positive (S+) pecks were reinforced on a VI 25 sec schedule, pecks at the other stimulus (S–) were never reinforced, nor pecks during the time out period The stimuli were presented in an irregular order, balanced to provide no more than 3 presentations in a row of one stimulus, and 6 presentations of each stimulus during each session. Half the animals in each condition were assigned the vertical stimulus as S+ and half

the horizontal on a priority basis. The number of pecks during each 1 min stimulus presentation of each discrimination session were recorded on counters and tabulated by the experimenter as the animal was performing. The animals were allowed 2 full days of rest in their own cages following the initial day of discrimination training, and then were run for one discrimination session per day until they reached a criterion level of 90% of all pecks on a given day occurring during S+ periods A lower limit of 100 total pecks to S+ and S– combined per session was established as a minimum for adequate performance Any animals who failed to reach that total on the first or second day of discrimination training were dropped from the experiment. If the pigeon exceeded 100 pecks on the first two sessions but failed to meet this level on any subsequent session, that single session was excluded from the analysis. However, only 2 animals out of 94 failed to meet the minimum performance criteria and were subsequently excluded from analysis.

Injection procedure. Injections were made intracerebrally, using a Kopf stereotaxic apparatus, fitted with pigeon ear bars and beak holder. A 26 guage hypodermic needle was mounted on the arm of the stereotaxic apparatus and connected through cannula fittings to 0.036 in diameter plastic tubing The tubing in turn was fit over the end of a 1 ml syringe which was mounted in a microburette apparatus (Micrometric Model SB2) which allowed controlled delivery of solution graded in microliters. Due to the thin and porous nature of the avian skull which offered virtually no resistance to our needle, injections could be made directly through the scalp and into the forebrain by simply mounting the animal in the stereotaxic apparatus and lowering the needle without drilling through the skull and without the use of anesthesia These injections caused no apparent disturbance to our animals All animals received injections into 4 sites on each side of the forebrain, with 10 μl of solution injected at each site The sites of injection were all in the dorsal portion of the forebrain, in the region of the hyperstriatum ventral and the anterio-dorsal portion of the neostriatum. (See [21] for more details.) Solutions were PM (18 μg/μl), or control solutions of physiologic saline. The entire injection (all 8 sites) procedure took approximately five min to complete, after which the animal was immediately returned to its home cage

Experimental design All animals were given identical pretraining and then given 1 day of discrimination training as described above Animals were randomly assigned to receive either PM or saline injections. They were removed from the training box and injected either immediately, 1 hour after, or approximately 24 hours following the initial training session Animals were then maintained in their home cages without further treatment (except for weighing, feeding and general observation) for 2 additional full days following the injection treatment On the next day (Day 2 of the training and chronologically the 4th day of the experiment, counting the initial training session and immediate injections as Day 1) discrimination training was resumed and the pigeons were run on discrimination training each day thereafter until they reached criterion as described above.

RESULTS AND DISCUSSION

Two aspects of performance were assessed: (1) change in percentage of pecks to S+ from session 1 to session 2 and (2) number of training days to reach criterion (DTC) (Fig. 1) Since the controls showed no differences in relation to time

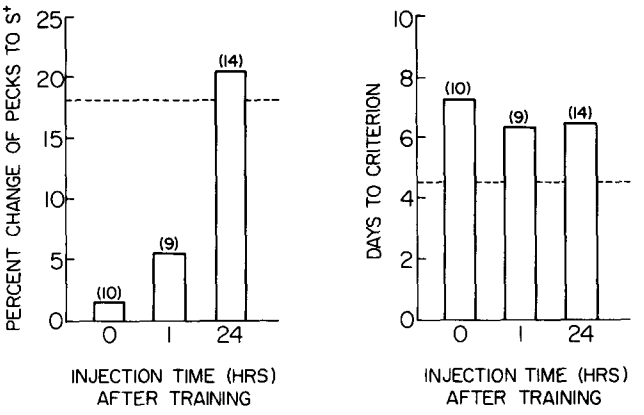

FIG 1 Mean change in percentage of pecks to S+ and mean days to criterion for birds injected with PM at different times after training The dashed line indicates the mean performance for the 24 saline controls, pooled over all injection times (n=26) Numbers in parenthesis indicate the number of animals in each PM group The mean ± SEM of each group are the following· For change in percentage saline (17 23 ± 2 17), immediate (1 6 ± 1 6), 1 hour delay (5 55 ± 4 82), 24 hour delay (20 43 ± 2 06) For days to criterion saline (4 5 ± 0 32), immediate (7 3 ± 0 62), 1 hour delay (6 33 ± 0 60), 24 hour delay (6 43 ± 0 81) One-tailed t test comparisons between each PM group and the combined saline controls for change in percentage yielded values of $p<0$ 005 for immediate injection, $p<0$ 01 for the 1 hour delay, and a negative t, insignificant, for the 24 hour group Similar comparisons for days to criterion yield values of $p<0$ 01 for the immediate group, $p<0$ 05 for the 1 hour delay and $p<0$ 02 for the 24 hour delay group

of injection, all controls were pooled for baseline comparison with the individual PM groups The mean increase of 18% for the controls during session 2 reflects their retention of Day 1 training; the immediate and 1 hour PM groups show little evidence of retention, whereas the scores of the 24 hour PM group indicate excellent retention. Thus, a very marked temporal gradient is clearly evident with respect to PM-induced loss of retention for initial discrimination training in the pigeon. When injected immediately following session 1 training, PM is highly effective in producing such loss, but injections 24 hours after training are totally ineffective.

The picture for DTC is different however, PM not only produces a "continued acquisition deficit" [21] over and above its amnesic effect when administered immediately or 1 hour after training, but a marked effect of this type persists even when injections occur 24 hours after the initial training In fact, the apparently greater effect of the *immediate* injections on DTC scores could be attributed to their initial amnesic effects, if the amnesic effect for one day of initial training is subtracted from the DTC scores, the continued acquisition effect is of the same magnitude in the immediate and 24 hour groups. The failure to find an amnesic effect with 24 hour delayed injections while still producing a continued acquisition deficit represents something of a paradox It implies that retention of the 1st day of discrimination *and* any within-day learning on the second day was normal whereas retention from the second day and/or within-day learning after the second day was impaired Suspecting some kind of statistical artifact, we examined the results for this group in detail, and found that indeed, the level of performance for

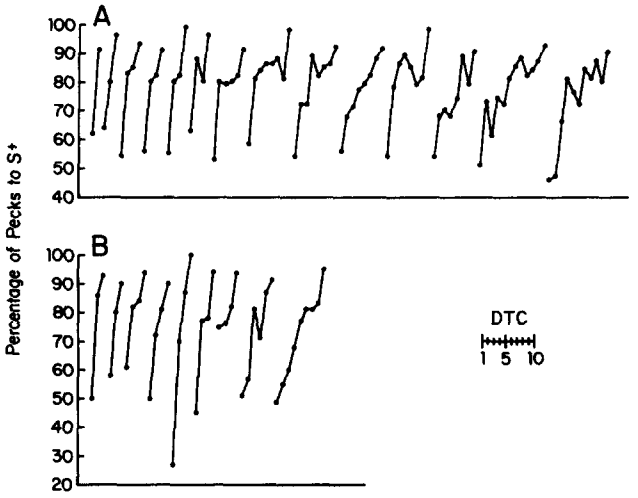

FIG 2 Percentage of pecks to S+ on each session for each animal injected 24-hours after the first training session with either puromycin (A) or saline (B) DTC represents days to criterion according to the scale on the right

the 24 hour PM group is essentially identical to that of the controls on the second day of training, but that these pigeons improve more slowly from that point on This is not a phenomenon produced by group averaging, examination of *individual* learning curves for all the animals in the 24 hour PM group as compared to controls indicates that the deficit is consistent from session 3 on, their S+ percentages tend to increase a small amount each day until criterion is reached, whereas controls continue to make large gains comparable to the increase in session 2 over session 1 (Fig 2)

Further, as might be expected, this small daily improvement appears somewhat unstable when one compares individual 24 hour PM curves to control curves. Reversions (a lower percentage of pecks to S+ on any given day than the day before) were quite rare in controls, occurring exactly once in only one of the 9 24-hour control animals, in contrast, 8 of the 14 24-hour PM animals showed at least one reversion, with a total of 13 such occurrences in this group This deficit, since it is extended over time and is temporally non-specific in our study, might be considered the result of "general debilitation" by PM treatment instead of a specific effect However, this is highly unlikely in the light of the normal performance of these animals on the first post-injection session and of our previous finding that this effect is *not present* when reward contingencies are reversed during post-injection trials [21].

These results confirm our previous observations that PM has two different effects upon discrimination learning in the pigeon and further indicates that the temporal parameters of these effects are different. In line with the results for goldfish [2, 5, 19] and 30 day-old-rats [4], the amnesic effects are completely absent when injections are delayed until 24 hours after training, providing some support for a consolidation-type interpretation of this aspect of PM's action. The continued acquisition effects, however, are still found when injections are delayed for 24 hours. The different temporal parameters of these two effects suggests that they are products of different mechanisms of action of PM Viewing PM's effects this way instead of searching for a unitary explanation may very well lead to a resolution of heretofore discrepant findings in the literature [3] on the behavioral effects of antibiotics

Flexner has suggested that the amnesic effects produced in rats and mice injected with PM 24 hours after training are due to the formation of peptidyl-puromycin fragments, formed when puromycin is incorporated into growing peptide chains which are then released in incomplete form from the ribosomes [7,13]. Since CXM inhibits protein synthesis at an earlier stage than PM, combined injection of both substances inhibits protein synthesis without leading to the formation of peptidyl-puromycin fragments, and it has been reported that such a combined injection attenuates the amnesic effect produced by PM injection 24 hours after training in mice [2,9]. We have found previously, however, that a combined injection of PM and CXM immediately after training attenuated *neither* the amnesia *nor* the continued acquisition deficit produced in pigeons by PM alone [21] This finding, coupled with the fact that our birds are not amnesic when PM injections are delayed until 24 hours after training, indicates that the "continued acquisition deficit" in pigeons may be mediated by mechanism(s) different from the underlying the amnesic effect found in mice when injections are given 24 hours or more after training. On the other hand, an alternative explanation for the apparent differences on retention between mice and pigeons with 24 hr delayed injections of PM may be related to task-specific aspects of training (i.e shock avoidance, appetitive, escape, etc.) and/or the type of performance assay used to evaluate retention [4].

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