

CHANGES IN SOCIAL BEHAVIOR AND BRAIN CATECHOLAMINES DURING THE DEVELOPMENT OF ASCORBATE DEFICIENCY IN GUINEA PIGS

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ABSTRACT

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Behavioral patterns of pairs of guinea pigs were recorded in 15-min observation sessions on alternate days during a 6-day baseline phase and a 21- to 25-day period following the introduction of an ascorbate-free diet. Ascorbate-deficient animals were compared to two pair-fed control groups. During the last 5 days of the experiment, marked reductions in frequency and/or cumulative duration of active behaviors (e.g. locomotion, rearing, social grooming) were observed in the ascorbate-deficient group, while duration of inactivity in proximity to the test partner increased greatly. The decline in probability of locomotion was greater at long temporal lags (> 15 sec) after locomotion of the test partner than at short lags. Evidence of behavioral changes began to appear after 9-13 days on the ascorbate-free diet. Assays of brain tissue after sacrifice on the last day of the experiment revealed significant reductions in concentrations of ascorbate and norepinephrine. Some behavioral measures were highly correlated with brain ascorbate but not with brain norepinephrine, suggesting that other transmitter systems are involved in mediating the behavioral changes. The results also suggest the value of measurement of social behavior in assessing the behavioral effects of dietary or other treatments.

KEY WORDS

Ascorbate - guinea pigs - social behavior - catecholamines

INTRODUCTION

Ascorbate deficiency produces a complicated pattern of behavioral and physiological changes (Hodges *et al.*, 1969). In

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humans, behavioral signs such as listlessness, weakness, and aversion to work are known to occur in scurvy. A controlled study of scurvy has shown deficits in human performance on certain psychomotor tasks, as well as personality changes, as assessed by the Minnesota Multiphasic Personality Inventory (Kinsman and Hood, 1971). Personality changes indicative of depression and withdrawal were among the earliest signs of scurvy. Moreover, many of the behavioral changes in this study were correlated with the measured deficiencies in the body pool of ascorbate.

These findings suggest that ascorbate levels might be abnormally low in some patients with depression, a possibility which has recently been confirmed (Schorah et al., 1983). Hospitalized depressed patients, as well as other categories of psychiatric patients, were shown to have significantly lower levels of plasma ascorbate than controls. These low ascorbate levels may have had etiological significance rather than being only consequences of a hospital diet since they were present even in recently admitted patients. Evidence that ascorbate, in combination with ethylenediaminetetraacetic acid (EDTA) is of therapeutic value for depression has also recently been reported (Kay et al., 1984).

These findings suggest the value of detailed experimental study of the behavioral effects of ascorbate deficiency. Such a study can be most readily conducted on guinea pigs, since they are the only common non-primate laboratory species which is unable to synthesize ascorbate from dietary constituents (Pauling, 1970). Though numerous studies of the effects of ascorbate deficiency in guinea pigs have been conducted, detailed quantitative observations of the behavioral changes which occur during a developing deficiency have not been reported, to our knowledge. Such observations are the basis of the present report.

The methods used to measure these changes involved direct observation of pairs of animals during regularly scheduled, timed sessions. This procedure allowed detection of changes in naturally occurring behavior, an approach which has been found useful in characterizing the effects of pharmacological agents (Mackintosh et al., 1977). Another feature of the method is that the behaviors occurred in a social setting, also an approach of

demonstrated value in assessing pharmacological effects (Niesink & van Ree, 1983; File and Hyde, 1978). It was thought that these methods might have similar value in measuring dietary effects on behavior. The use of a social situation may be especially important if one regards these guinea pig behaviors as a model of the psychopathology associated with ascorbate deficiency. Ellison (1979) has argued persuasively for observation of social behavior in potential animal models of psychopathology since behavior in social situations is a sensitive indicator of such disorders in humans.

Current understanding of the biochemistry of depression attributes some role to brain norepinephrine systems. Generally, either pharmacological or psychological treatments which deplete norepinephrine in the brain tend to induce depressive behaviors (Anisman and Zacharko, 1982). Since there is evidence that ascorbate deficiency also depletes brain norepinephrine (Deana et al., 1975; Saner et al., 1975), we sought to determine whether brain norepinephrine depletions would accompany the behavioral changes.

METHODS

Subjects

The subjects were 24 male guinea pigs (Camm Research Lab Animals, Wayne, New Jersey), housed individually and weighing approximately 200 g each upon arrival in the laboratory.

Procedure

Diets. All animals were immediately given the control diet (ascorbate-free guinea pig diet from ICN Nutritional Biochemicals, Cleveland, Ohio, with 2 g of ascorbate added per kg/diet) for an adaptation period of 15 days as well as for the 6 days of the baseline phase of the study (see "Behavioral Testing"). After this period the animals were randomly assigned to the following three dietary groups of 8 animals each:

- (1) Ascorbate-deficient group--ascorbate-free diet mentioned above;
- (2) Pair-fed control group--control diet described above;
- (3) Tyrosine-supplemented, pair-fed control group--control diet but with 10g of tyrosine added per kg diet.

Pair-feeding was employed because of the possibility that behavioral or biochemical differences might otherwise result from reduction in total food intake rather than from the absence of ascorbate per se. This procedure consisted in offering on a

given day to each of the pair-fed animals only the mean weight of food eaten by the ascorbate-deficient group on the previous day. Tyrosine supplementation was employed in one pair-fed group to control against the possibility that the reduction in total intake produced behavioral or biochemical effects partly through decreasing tyrosine intake (Lehnert *et al.*, 1984).

Behavioral Testing: Baseline Phase. During the six days immediately preceding the introduction of the diets, baseline behavioral observations were conducted. Animals were observed in pairs which had been matched for weight. These same pairings were used for all behavioral test sessions.

The pairs were observed under low illumination through a one-way mirror during the first four hours of the light phase of the lighting cycle. At the beginning of a test session a pair was placed in an aquarium (26.5 X 51 X 30.5 cm high) and allowed a 10-min adaptation period. The behaviors of both animals were recorded for the next 15 min by means of a keyboard linked to a microcomputer. All test sessions were conducted by the same observer. Animals were kept in individual cages between tests.

Onsets and offsets of each defined category of behavior were recorded and stored on diskette for later analysis. On the basis of previous ethological study of the guinea pig (Rood, 1972) and our own pilot work, the following nine mutually exclusive and exhaustive categories of behavior were defined:

- Locomotion--taking two or more steps in any direction;
- Sitting together--sitting within approximately 2.5 cm of the behavioral test partner;
- Sitting alone--sitting at least 2.5 cm away from the partner;
- Self-grooming--licking, scratching, nosing, nibbling, combing, or wiping the face;
- Social grooming--above grooming activities directed at the partner;
- Aggressive behavior--head thrusting, attacking-lunging, or threatening;
- Comfort movements--yawning, stretching, or shaking;
- Sexual behavior--nasal-anal contact, chin-rump contact, following, or mounting;
- Rearing--rising on hindlegs to an angle with the floor of more than 45 degrees.

Animals were observed on alternate days, half of them (6 pairs) being observed each day. Hence, three complete sets of test sessions were conducted during the 6-day baseline phase.

Behavioral Testing: Experimental Phase. The experimental phase of the study commenced with the introduction of the ascorbate-deficient and tyrosine-supplemented diets. Behavioral observations continued throughout this phase on alternate days as during the baseline phase. The pairings of animals were the same as during the baseline phase, and the two animals of a pair

were always from the same dietary group. When the ascorbate-deficient animals appeared near death, they were sacrificed. The members of a pair were sacrificed on the same day together with the lowest-weight pair of animals from each of the pair-fed groups. The final behavioral test sessions were conducted 2-3 hours before sacrifice; in some instances this session was 1 day after the previous session (rather than the usual 2 days). This procedure resulted in sacrifice on the 21st day of the experimental phase for one pair per group, on the 23rd day for two pairs per group, and on the 25th day for 1 pair per group. The behavioral data reported for the experimental phase are for the last 11 observations in all animals regardless of the day of sacrifice.

Biochemical Measures. Upon sacrifice a blood sample was collected and centrifuged after coagulation, and the serum was placed on ice. The brain was rapidly removed, bisected by a mid-sagittal cut, and stored on dry ice. Within 6 hours of sacrifice, both the blood serum and one half-brain were assayed spectrophotometrically for ascorbate content (Omaye et al., 1979). The other half-brain was assayed fluorometrically for norepinephrine and dopamine within 4 days of sacrifice (Shellenberger and Gordon, 1970).

Data Analysis. The biochemical data were analyzed by means of an analysis of variance with planned contrasts between the ascorbate-deficient group and the mean of the other two groups (Myers, 1979). The possibility that norepinephrine or dopamine levels might be elevated by tyrosine supplementation could be rejected simply by examination of the group means (Table 1). The absence of a biochemical difference between the two pair-fed groups together with the lack of a consistent pattern of differences between them on any of the behavioral measures led us to combine these groups in all subsequent analyses.

The frequency and cumulative duration for each behavioral category in each animal were extracted from the data file for each test session. Since the scores for the members of a pair tested together could not be assumed independent, the means for a pair were calculated and used in all subsequent analyses. This procedure, though perhaps conservative, was necessary because of the sensitivity of multivariate analysis of variance to violations of the independence assumption (O'Brien and Kaiser, 1985).

The mean frequencies and cumulative durations for a pair of animals were analyzed separately for each behavioral category using a multivariate analysis of variance approach (O'Brien and Kaiser, 1985) with planned comparisons of the mean of the three sessions of the baseline phase with each of the last seven sessions (about the last two weeks) of the experimental phase. In effect, this procedure ignores the data for the first 7-12 days of the experimental phase, a conservative estimate of the time necessary for deficiency signs to develop, at least in earlier observations on food intake and body weight in our

laboratory. The probability of a Type I error was controlled within each set of comparisons at the .05 level by the Bonferroni procedure. Thus, the probability necessary for significance of a comparison was .00714 since seven comparisons were conducted in each set. The between-subjects portion of the multivariate analysis involved a contrast between the ascorbate-deficient group and the mean of the two pair-fed groups. In brief then, the analysis of the frequency and duration data compared the ascorbate-deficient group with the two control groups; the comparisons concerned changes from baseline in each of the last 7 test sessions.

In a further effort to differentiate the experimental group from the pair-fed groups, sequences of behavior were also examined. The possibility of sequential dependencies between the behaviors of a pair of animals was first examined crudely by calculating Pearson correlation coefficients between frequencies for the two members of the pairs of animals. All possible pairs of behavioral categories were examined separately in each of the three groups. For each pair of behavioral categories a single correlation was calculated within a group across all pairs of animals and across all 14 test sessions of the experiment.

Lag sequential analyses were conducted for some of these pairs of categories, depending on the results of the correlational analysis (Bakeman, 1978). A lag sequential analysis determines the conditional probability of a behavior at various intervals after some criterion behavior. Software was written to divide a test session into short intervals (5-second intervals were arbitrarily chosen for the present study) and then to record the occurrence or non-occurrence of selected behaviors. The program allows selection of a criterion behavior in one animal of the pair. Then it records whether a selected behavior of the second animal occurs during the same 5-sec interval (lag 0), the following interval (lag 1), the interval after that (lag 2), etc. From these occurrences it calculates the proportion of instances of the criterion behavior for which the behavior of the second animal occurs at a particular lag. These conditional probabilities were summarized as group conditional probability profiles for each test session. These profiles show changes in conditional probability across lags.

These profiles were analyzed separately for each test session by multivariate analysis of variance. Again the between-subjects portion of the analysis utilized a contrast between the ascorbate-deficient group and the mean of the remaining two groups. The differences in the profiles were assessed by planned linear and quadratic trend contrasts (O'Brien and Kaiser, 1985). Overall Type I error rate was controlled at the .05 level within each test session by the Bonferroni procedure. Thus, since this analysis involved only two trends, the probability necessary for significance was .025.

RESULTS

Frequencies and Cumulative Durations of Behavior.

Significant differences between the ascorbate-deficient and pair-fed groups are clearest and most consistent for two categories of behavior: locomotion and sitting together. Smaller but nonetheless significant differences were also found for grooming and for rearing behaviors. No other significant differences from control groups were found.

The duration of locomotion (Figure 1) in the control groups changes little through the baseline and experimental phases of the study. In contrast the locomotion of the ascorbate-deficient group declined from the level of the baseline phase. The latter pattern was significantly different from that of the controls in sessions thirteen ($F(1,9) = 19.034, p = .002$) and fourteen ($F(1,9) = 38.095, p < .001$) of the experimental phase. Similarly, the frequency of locomotion (Figure 1) appears to have increased slightly in the control groups throughout the 14 test sessions, while in the ascorbate-deficient group it declined markedly in the last three test sessions of the experimental phase. These two patterns were significantly different in sessions twelve ($F(1,9) = 20.110, p = .002$), thirteen ($F(1,9) = 28.265, p < .001$) and fourteen ($F(1,9) = 56.180, p < .001$).

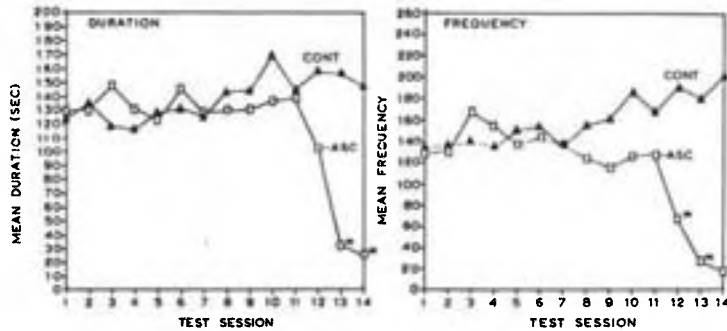


Fig. 1. Mean cumulative duration (left) and frequency (right) of locomotion in ascorbate-deficient (-ASC) and combined control (CONT) groups. Test sessions 1-3 = baseline phase and test sessions 4-14 = experimental phase of the study (further described in the text). Asterisks indicate significant group differences in changes from baseline (see "Data Analysis").

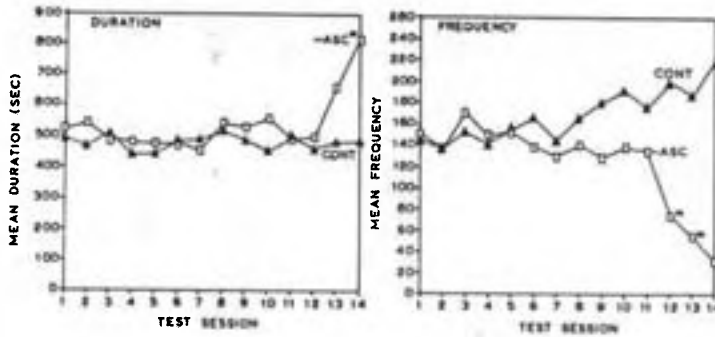


Fig. 2. Mean cumulative duration (left) and frequency (right) of sitting together in ascorbate-deficient (-ASC) and combined control (CONT) groups. Other conventions are as for Fig. 1.

Animals on the ascorbate-deficient diet showed an increase in the duration of sitting together in late test sessions (Figure 2), and this increase was significantly different from the lack of change seen in the control groups on test session fourteen ($F(1,9) = 54.786, p < .001$). On the other hand, differences in the frequency of sitting together can be noted relatively early in the experimental phase of the study (Figure 2). Here the decline from baseline was significantly different from the pattern of the controls in test sessions twelve ($F(1,9) = 24.467, p = .001$), thirteen ($F(1,9) = 40.341, p < .001$), and fourteen ($F(1,9) = 81.702, p < .001$).

Thus, as ascorbate deficiency developed in the animals, the frequency of sitting together changed first, and later both frequency and duration were affected. In other words, the number of episodes of this behavior was declining, but their mean duration was increasing. It is noteworthy that the ascorbate-deficient animals were engaged in sitting together for about 90% of the time in the last test session of the experiment, while the same behavior occupied about 55% of the time in the sessions of the baseline phase (Figure 2).

The frequency of social grooming increased gradually across the 14 sessions of the experiment in the control groups (Figure 3). This increase from the baseline phase of the experiment was significantly different from the change seen in the ascorbate-

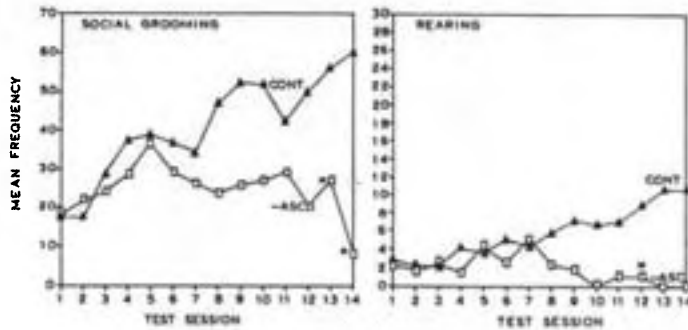


Fig. 3. Mean frequency of social grooming (left) and of rearing (right) in ascorbate-deficient (-ASC) and combined control (CONT) groups. Other conventions are as for Fig. 1.

deficient group at test sessions thirteen ($F(1,9) = 19.140$, $p = .002$) and fourteen ($F(1,9) = 13.484$, $p = .005$). A similar pattern for duration of this behavior did not reach significance.

The mean frequency of rearing behavior (Figure 3) in ascorbate-deficient animals was lower than in controls in all test sessions from eight to fourteen. The changes from baseline were significantly different only in test session twelve ($F(1,9) = 29.757$, $p < .001$). However, the lack of significance for sessions thirteen and fourteen may be partially due to a severe violation of the homogeneity of variance assumption of the test since all the scores for the ascorbate-deficient group were at 0 for those two sessions (hence, also had a variance of 0). A Mann-Whitney U-test applied to the changes from baseline does reach significance at the conventional .05 level for both these test sessions though not at the more stringent level employed for the other analyses reported here (thirteen: $U = 1$, $p < .01$; fourteen: $U = 4$, $p < .05$). The pattern for mean duration of rearing behavior was similar to the mean frequency of that behavior, but none of the differences between ascorbate-deficient and control groups attained significance.

It is important to observe that the various types of behavior for which significant differences were found account

for a large proportion of the time of the session--approximately 75%. All but one of these behaviors, rearing, showed mean durations of a minute or more for most sessions. Thus, the behaviors with significant group differences are those which were more extensively and, hence, probably more reliably sampled. In addition, nonsignificant declines from baseline were observed in the ascorbate-deficient group in all the remaining categories of behavior.

Sequences of behavior.

The calculation of Pearson correlations between frequencies of pairs of behaviors, one in each animal, revealed only the following pairs with correlations consistently greater than or equal to .7 (i.e., about 50% of the variance accounted for): sitting together in one animal and locomotion in the other, sitting alone in one and sitting alone in the other, and locomotion in one and locomotion in the other. These pairs of behaviors were then selected for lag sequential analysis.

This analysis for sitting together and locomotion produced very flat conditional probability profiles, i.e., very little evidence of change in conditional probabilities across lags. Thus, there did not appear to be sequential dependence between these two behaviors.

A lag sequential analysis for sitting alone in one animal and sitting alone in the other showed a very sharp decline in conditional probability from lag 0 to lag 2 (from $>.8$ to $<.4$). Thus, the occurrence of this behavior in one animal appeared to affect its likelihood in the other. However, the conditional probability profiles were almost identical for the three groups of animals.

The lag sequential analysis for locomotion in one animal and locomotion in the other showed group differences which are displayed for the last two test sessions in Figure 4. Note the sharper decline across the first few lags in the ascorbate-deficient group than in the combined control groups. This pattern resulted in significant differences in linear ($F(1,9) = 30.364, p < .001$) and quadratic ($F(1,9) = 14.068, p = .005$) trends for test session thirteen and in a significant difference in linear trend in session fourteen ($F(1,9) = 9.103, p = .015$). The differences in test session twelve or any of the earlier sessions did not attain significance. These profiles (Figure 4)

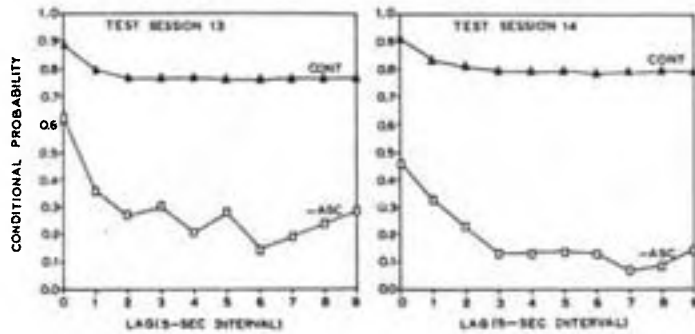


Fig. 4. Mean lag sequential probability profiles for locomotion in ascorbate-deficient (-ASC) and control (CONT) groups in test sessions 13 (left) and 14 (right). Plotted on the y-axis is the probability of locomotion in one animal given the occurrence of locomotion in the other during the 5-second interval marked 0. See text for statistical analysis.

also clearly show lower probabilities at all lags for the ascorbate-deficient group. Though a statistical test of this difference can be done, it adds little to what is already known from the analysis of the frequency and duration of locomotion.

Biochemical measures.

Means for each of the three groups of animals on each biochemical measure are presented in Table 1. As expected, the ascorbate-deficient group had significantly lower serum ascorbate ($F(1, 21) = 30.30, p < .001$) as well as lower brain ascorbate ($F(1,21) = 139.95, p < .001$) than did the combined control groups. Significantly lower concentrations of brain norepinephrine were also demonstrated in the ascorbate-deficient group as compared to the control groups ($F(1,21) = 4.70, p = 0.042$). However, there was no significant difference between groups in the concentration of brain dopamine.

DISCUSSION

The behavioral data demonstrated a number of changes that appear during the development of ascorbate deficiency. Several active behaviors, particularly locomotion, occurred less and less frequently and for a declining cumulative duration. During the last two or three test sessions this change was

TABLE 1

The effect of dietary ascorbate deficiency on concentrations of serum and brain ascorbate, and on brain catecholamines (means and standard errors).

Group	Pair-fed Control	Tyrosine supplemented Control	Ascorbate-Deficient
Serum Ascorbate (ug/ml)	1.91 \pm 0.35	1.43 \pm 0.18	0.11 \pm 0.05**
Brain Ascorbate (ug/gm wet wt.)	22.90 \pm 1.34	25.20 \pm 1.24	8.65 \pm 0.28**
Brain Nor-epinephrine (ug/gm wet wt.)	0.94 \pm 0.13	0.83 \pm 0.11	0.57 \pm 0.11*
Brain Dopamine (ug/gm wet wt.)	0.94 \pm 0.05	0.93 \pm 0.07	0.85 \pm 0.06

*p < .05, **p < .001 compared to the combined control groups by planned comparison procedure.

accompanied by fewer but longer episodes of sitting together. By the fourteenth session the ascorbate-deficient animal's repertoire had shrunk to little more than a few very long bouts of sitting in proximity to the test partner.

Of course, it is not surprising that the animals should have become inactive as they developed a severe deficiency disease. What is perhaps not so obvious is that the animals should have preferred to be near the test partner for these long inactive periods. Guinea pigs are known to freeze when placed in a novel environment (Suarez and Gallup, 1982), and one might wish to argue that this tendency is heightened by the deficiency state. However, the reduction in locomotion observed here was occurring as the environment actually became more familiar to the animals. If the ascorbate-deficient animals were simply freezing more, then one would have expected them also to spend more time sitting alone, but this behavioral category actually showed nonsignificant decreases in frequency and duration in the last few test sessions. Of course, it is hazardous to infer the motivation for the preference to be near the test partner. Perhaps it is similar to that for the increased affiliative

behavior observed in rats during noise stress (Taylor, 1981). Or perhaps it is no more than seeking a warm object to help maintain body temperature.

The changes which occurred generally attained significance, by the criterion employed here, only in the last 3 test sessions, i.e. during the last 5 days, before sacrifice. However, examination of Figures 1-3 shows that on several measures the patterns of means for the ascorbate-deficient and control groups diverged as early as the eighth session (almost two weeks before sacrifice or 9-13 days after the introduction of the deficient diet). It is possible that larger sample sizes would have led to significant differences at these earlier points also. This divergence occurred as early as or slightly before the decline in food intake which we observed in carrying out the pair-feeding procedure. Thus, the present results appear consistent with finding of early behavioral changes in the development of experimental scurvy in humans (Kinsman and Hood, 1971). These results also suggest the possible value of our procedures in producing a model for investigation of mechanisms underlying the early behavioral changes of ascorbate deficiency.

The lag sequential analyses cast further light on the nature of the social interactions in this test situation and the changes which occurred with developing ascorbate deficiency. These analyses revealed social modulation of some of the behaviors observed. The strongest social influence was on sitting alone, a category which might at first be assumed entirely nonsocial. However, in the test situation employed for this study, the animals showed a rather strong tendency to display this behavior at about the same moment in a test session. This tendency was similar in all three groups of guinea pigs.

One animal of a pair also displayed locomotion during the same interval as his test partner (lag 0) with somewhat greater probability than at other times (Figure 4). This trend across lags was significantly enhanced in the ascorbate-deficient animals. It is as though the animal retained some ability to respond with locomotion to this same behavior in his partner in spite of the massive reduction in the overall likelihood of this behavior. The social stimulus to locomotion appears to remain effective somewhat further into the development of the deficiency state than do other stimuli for this behavior. These findings suggest that the lag sequential technique may be useful in detecting social-behavioral effects of other experimental treatments also.

Ascorbate concentrations in both the blood serum and the brain were significantly reduced in the ascorbate-deficient animals, as expected (Table 1). The percentage reduction relative to controls was less for the brain than for serum, as has previously been reported (Hughes et al., 1971). Also consistent with previous reports was the significant reduction in brain norepinephrine concentration in ascorbate-deficient animals (Deana et al., 1975; Saner et al., 1975). The absence of effect on dopamine concentration was not unexpected since both increases and decreases in this parameter had been found in previous studies (Deana et al., 1975; Saner et al., 1975).

The present study also permits the correlation of behavioral measures with biochemical ones since all were obtained in the same animals. These correlations would be expected to be greatest for test session 14 because sacrifice occurred shortly after this session. Brain ascorbate concentration showed significant correlations with several of the behavioral categories. These correlations were greatest for locomotion and sitting together. With these categories the Pearson correlations were greater than .8 for both frequencies and durations ($p < 0.001$; r negative in the case of duration of sitting together). Thus, the results are consistent with the simple hypothesis that the behavioral effects of an ascorbate-deficient diet are mediated by a reduction in brain ascorbate.

The role of brain norepinephrine in mediating the behavioral changes is less clear. Pearson correlations with the behavioral data from test session 14 were not significant for any category. The largest correlations, again for locomotion and sitting together, ranged between .3 and .4. Thus, it appears either that other measures of the norepinephrine system (e.g. turnover, receptor number, etc.) are required or that other transmitter systems are more directly involved in mediating these behavioral changes. Thus, the behavioral characteristics of ascorbate-deficiency, which may be depression-like, are probably not mediated in a simple fashion by norepinephrine systems any more than are other forms of depressive behavior (Anisman and Zacharko, 1982).

In summary, the present study has provided a description of the social-behavioral changes which occur during the development of ascorbate deficiency in guinea pigs. The main features of these changes were reductions in several active behaviors and an increased prevalence of long bouts of sitting together. The

probability of locomotion declined to near 0 except within 10 or 15 seconds of locomotion by the test partner. Some of the changes observed were highly correlated with brain ascorbate concentrations but only slightly correlated with brain norepinephrine concentrations.

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