EFFECTS OF EARLY HANDLING AND EARLY MALNUTRITION ON DEVELOPMENTAL CHANGES IN THE OPEN FIELD BEHAVIOR OF RATS

by

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Abstract

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In attempting to determine whether nutritional status and handling during the first three weeks of postnatal life affect activity in the open field and neurochemical changes in rats, the present study has perhaps raised as many questions as it has answered. The questions that have been answered are fairly straightforward. Early handling results in an increase in the amount of activity in the open field both immediately post-weaning and during early infancy. The long-term effects of handling during the nursing period are also evident in most open field behaviors when testing is carried out for the first time in adulthood. The effect of nursing from a dam on a low-protein diet during early life on the amount of activity of rat pups in the open field during early development and in adulthood is in the same direction as that of handling, with an apparently stronger influence on the activity of those animals that were handled coincidentally with the deficient diet.

Early dietary deficiency and early handling also exert a profound and seemingly permanent effect on the number and size of the cells in rat forebrain. As has already been demonstrated by other investigators, dietary deficiency during the lactation period results

in a smaller number of cells of normal size when compared to adequately nourished controls. Early handling similarly results in a smaller number of cells when measurements are taken at 55 days of age. However, these cells are larger in size when compared to non-handled controls.

Future studies should test open field behavior and perform neurochemical analyses on a few animals from each group sequentially during development rather than at a single point in ontogeny in order to determine whether there is an effect of the experimental manipulations on the behavioral and neurochemical development. The behavioral and chemical measures should be carried out concurrently in order to determine the relationship between behavioral functioning and central nervous structure, as well as to determine whether any changes in the differences among the experimental groups with change in age on one of the measures would be mirrored by changes with age on the other measure. The use of more than one behavioral measure would also provide additional information and analytical tools.

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INTRODUCTION

The present investigation is a longitudinal developmental study of the effects of both early malnutrition and early handling on ontogenetic changes in open field behavior in rats. The study was designed to determine: 1) the effects of malnutrition during the lactation period on behavior in the open field; 2) the effects of handling during the lactation period on the same behavior; 3) the relationship between early nutritional status and early handling in determining such behaviors; and 4) the nucleic acid responses in the brains of malnourished[1] and handled animals following five weeks of dietary rehabilitation.

Until relatively recently, medical belief concerning nutritional deficiency generally held that prenatal malnutrition "spared" the fetus. The fetus and the gestational female were considered to have a parasitic relationship so that the fetus would extract all necessary nutrients from maternal stores, even at the expense of maternal nutritional status (Thomson, 1968). It was also commonly believed that postnatal malnutrition "spared" the brain. Although it was obvious that physical growth was adversely affected by nutritional

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1. The term malnutrition means any deviation from normal, adequate nutrition (McLaren, 1972). In the present paper, the term malnutrition always refers to some form of undernutrition --- either starvation or semi-starvation, or a deficit of one or more nutrients, particularly protein.

insult, the brain was somehow thought to be differentially protected (Lehr and Gayet, 1963).

The permanent physical stunting of poorly nourished populations has long been recognized (Boas, 1910, cited in Birch and Gussow, 1970; Greulich, 1958) and the possibility of intergenerational effects on physical stature has even been considered (Boas, 1910, cited in Birch and Gussow, 1970). Changes in behavior following early malnutrition have also been noted, although prior to the last two decades, these changes were believed to be transient. Thus, as early as 1933 and 1935, Williams reported that young children diagnosed as having Kwashiorkor[1] exhibited extreme apathy to their surroundings. They were said to seek the least stimulating environment possible --- dark and warm places --- and would remain there until moved. According to Williams, a renewal of interest in environment was indicative of recovery. Therefore, the "mental changes" were considered to be

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1. The term Kwashiorkor, meaning "The disease the deposed baby gets when the next one is born" (Williams, 1935 p. 1151), was introduced into medical language from the Ghanaian language. It was believed by the Ghanaians to be a mysterious demonstration of jealousy by the first child in response to the imminent birth of another child. However, coincident with the next pregnancy is the cessation of lactation and weaning of the first child onto the adult carbohydrate-rich and protein-poor diet, resulting in a nutritional deficiency. There is another protein-calorie deficiency disease --- Marasmus --- defined as a near starvation level of intake of all nutrients in infants who, 1) are nursing from mothers with inadequate supplies of milk (perhaps due to a maternal protein deficiency), 2) have been weaned early onto a diet of watered-down nutrients, or 3) have been fed a diet of watered-down nutrients since birth.

merely symptomatic with no more significance than any other temporary symptom.

Although there is evidence suggesting only transient mood changes in adults subjected to six months of semistarvation (Keys, Brozek, Henschel, Michelson, & Taylor, 1950) this does not seem to be true for infants and young children. Recent evidence casts doubt on the traditional belief that developing and mature organisms react in similar ways to dietary inadequacy. Although the adult central nervous system appears to be remarkably resistant to long-term alteration even in the face of severe starvation (Dobbing, 1968), the developing central nervous system is indeed vulnerable to nutritional insult.

While the physical consequences of early malnutrition have been fairly well documented, interest in the functional consequences of early malnutrition is a recent phenomenon which derives from a concern with the behavioral development of approximately one in every three children born in the world whose diets during the first months and years of life have failed to supply them with nutrients necessary for normal growth and development. Numerous studies (Barrera-Moncada, 1963, cited in Cravioto and Robles, 1965; Botha-Antoun, Babayan, & Harfouche, 1968; Brockman and Riccuitti, 1971; Cabak and Najdanvic, 1965; Champakam, Srikantia, & Gopalan, 1968; Chase and Martin, 1970; Cravioto and Robles, 1965; Mönkeberg, 1968; Pek, Tjiok, Qey, & Lauw, 1967; Richardson, Birch & Hertzig, 1973; Stoch and Smythe, 1963, 1967) have reported that children who had experienced

malnutrition during early life performed subnormally on standardized psychological tests, demonstrated poor school performance, poor language development, personal and social maladaptations, as well as a variety of other abnormalities when tested several months or even years later.

Interpretation of the above findings in terms of the relationship (correlational or causal) between early malnutrition and psychological development is severely hampered for a number of reasons. First, malnutrition never occurs in isolation, but is invariably only one factor in a complex, multi-faceted at-risk environmental setting including medical, economic, social-familial, educational and genetic factors, each of which is known to adversely affect physical and psychological development. In order to assess the contribution of nutrition independent of these other factors, it would be necessary to hold all the other factors constant. Ethical constraints prohibit the use of these necessary controls and the manipulation of variables which would enable assessment of the sequelae of dietary inadequacy independent of other concommitant at-risk life conditions and circumstances. Therefore, study of the behavioral effects of early malnutrition in human populations is, of necessity, limited to naturally-occurring clinical situations which yield correlational information. The severity, duration and age of onset of nutritional inadequacy is difficult, if not impossible, to systematically assess in such field studies. Moreover, follow-up studies of the original sample of children are hampered by continually

changing housing patterns, social and physical mobility, and repeated illness. An additional difficulty derives from the fact that standardized tests have been the most widely used tool to assess behavioral retardation in malnourished populations. Since such tests are imperfect predictors of functioning in later life in any population, and since most of these tests have been developed and standardized on different populations from those on which they are used, the validity of the results of these studies may be seriously questioned. Because of the various ethical constraints and methodological difficulties, interpretation of the results of field studies is problematic, and attention has turned to the use of animal models in the study of the behavioral consequences of early malnutrition.[1]

Although there are several problems in the use of animal models to examine the effects of nutritional deficiencies, their use permits the experimental control of variables that is difficult if not impossible in human studies. For example, the age of onset, the duration,

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1. There have been some studies published since the completion of the present study in which the later behavior of early malnourished children was investigated without the possible influence of adverse non-nutritional variables (Ellis and Hill, 1975; Lloyd-Still, Hurwitz & Wolff, 1974; McLaren, Yatkin, Kanwati, Sabbagh & Kadi, 1973; Stein, Zusser, Saenger & Marolla, 1972; Winick, Meyer, & Harris, 1975). In general, these studies have found that if the malnourished child is removed from his original environment early enough, and if the subsequent environmental and nutritional support is long-term, even though height and weight may still be subnormal, performance in school and on standardized intelligence tests are undistinguishable from that of well-nourished children in the same environments.

and the severity of nutrient deprivation can be experimentally manipulated. The biochemical and behavioral sequelae can be systematically studied under specific conditions of rearing and testing. Finally, several factors in the environment which interact or covary with malnutrition in the human situation --- such as infection (Scrimshaw, Taylor & Gordon, 1959, 1968; WHO, 1965), perinatal mismanagement, or loss of learning time during the illness (Birch, 1972) --- known to independently affect both brain development and behavior can be controlled and systematically investigated using animal models. While definitive answers to questions concerning humans can only come from studies of humans themselves, malnutrition does affect basic central nervous system processes and adaptive function in all mammalian organisms.

Although basic biochemical processes are common to all mammalian species, the organization and developmental course of these processes is species-related, resulting in different functional outcomes in different species. It is therefore possible to generalize about biochemical processes from lower level to higher level mammalian organisms, but it is not possible to generalize about specific behavioral functions. Thus, experimental intervention at the basic biochemical level, such as early malnutrition, results in similar biochemical deviations in different species. However, such intervention results in different behavioral adaptations in different species. Therefore, the effects of biochemical changes on specific adaptive behavior in any one species cannot be generalized to any other

species. Specifically, the fact that early malnutrition in rats results in changes in certain behaviors (e.g., open field activities) cannot be generalized to learning, intellectual, or social deficits in humans subjected to nutritional deprivation early in life. It can only be concluded from the results of laboratory studies in rats and field studies in humans that early nutritional deficiency alters particular aspects of behavioral functioning in rats as well as in humans.

Early malnutrition and the CNS

Malnutrition during early life has detrimental effects on the developing nervous system. The degree of vulnerability of the brain to nutritional insult at any particular point in ontogeny is uniquely determined according to the phase of cellular development that is taking place at the time. All organs and tissues go through the following three phases of cellular development: 1) hyperplasia; 2) hypertrophy and hyperplasia; and 3) hypertrophy alone. The transition from phase to phase is a function of the rate of DNA synthesis and its relation to protein synthesis and occurs at differential rates in different species. Accordingly, during the first phase, the rate of DNA synthesis is at a maximum and any accretion of protein is utilized in the formation of new cells. During the second phase, the rate of DNA synthesis is slowed while the rate of protein synthesis is maintained, resulting in a slower rate of increase in cell number as well as an increase in cell size. During the final phase, no further DNA synthesis takes place, terminating growth by

cell number. A net increase in protein synthesis, however, permits further growth in cell size.

The nature of structural damage and of consequent behavioral dysfunction, as well as the prognosis for recovery following nutritional insult depends upon the phase of cellular development during which the insult occurred. Whereas inhibition of cellular development during the hyperplastic period is likely to result in a permanent reduction in cell number, such inhibition during the hypertrophic period is likely to result in a temporary reduction in cell size. In addition, nutritional insult during more than one phase of cellular development is likely to result in greater inhibition than during the individual phases alone (Winick and Rosso, 1969a).

Since the time course of cellular development varies from species to species, it is necessary to know the time periods during which given processes are occurring for any given species in order to enable the assessment of the biochemical and structural consequences of nutritional insult. For example, in whole human brain, the rate of DNA synthesis is highest just prior to birth, continues at a negatively accelerating pace until between six to eight months, and ceases altogether between one and two years of age although protein synthesis continues throughout the entire growing period (Winick and Rosso, 1969b). In the rat brain, on the other hand, the rate of DNA synthesis in whole brain is highest ten days postnatally, then begins to slow, and finally stops at 17 days postnatally, while net protein synthesis continues until approximately 29 days of age (Winick and

Noble, 1965). It should also be noted that the three basic phases in cellular development do not occur uniformly throughout the whole brain; there are regional variations (Fish and Winick, 1969a, 1969b) as well as variations among cell types (Dobbing, 1972). Those regions or cell types which are most actively dividing at any particular time are most affected by nutritional or other biochemical insult.

Winick and Rosso (1969a; 1969b) performed postmortem analyses on the brains of nine infants who died of severe marasmus during the first year of life in Santiago, Chile, as well as the brains of a control group of 31 infants who died of accidental causes in the United States (Winick, 1968) and a second control group of 10 infants who died accidentally in Santiago. Whereas the brains of the normally nourished children from Chile were comparable to those of the United States children in weight, total protein, RNA, and DNA, the brains of the nine infants who died from severe malnutrition were proportionately lower in all these measures. Although the brain cells of the malnourished infants contained the normal amounts of protein and RNA, the number of cells in various anatomical loci was drastically reduced in comparison to the normals. This reduction in cell number was more pronounced in cerebrum and cerebellum than in brainstem, reflecting differences in the timing of cellular growth in different parts of the brain.

A number of experimental studies of the effect of early malnutrition on cellular growth and multiplication in the CNS in subhuman

animals have yielded results similar to those obtained in human infants and children. Winick and Noble's (1966) investigation of the cellular response of rat brain to 21 days of nutritional deficiency at different times during postnatal development demonstrated that malnutrition during the lactation period[1] resulted in a proportional reduction in RNA, DNA, and protein. This indicated a smaller than normal number of normal sized cell, and was not reversed with refeeding to 133 days of age. Malnutrition during either the first or the second 21 days following weaning[2] resulted in a proportional reduction in RNA, protein, and the weight of the brain, but no change in DNA content. This indicated a deficit in cell size when compared to normals with no effects on cell number, and was reversed with realimentation to 133 days of age.

In summary, the deleterious effects of early malnutrition on the developing central nervous system are indisputable. Nutritional deficiency during the periods of cell division or myelinization results in long-term and possibly irreversible curtailment of these ongoing processes. The effects of nutritional deficiency after these periods appears to be more transient. Thus, one of the crucial factors determining the consequences of suboptimal dietary intake is the time period(s) during development in which inadequate nutrition

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1. An increased litter size technique was used to accomplish malnutrition during the lactation period.

2. Caloric restriction of about one half the estimated daily requirement was used to accomplish malnutrition following weaning.

takes place. The nature of the dietary insufficiency, its duration, and the duration of nutritional rehabilitation, as well as social, familial, and medical factors may also be important in determining the central nervous system sequelae of early malnutrition (Birch, 1972).

Early Malnutrition and Behavior

Early postnatal malnutrition in animal species is most commonly accomplished by using either the large litter technique or by restricting the dam's food intake. It could also be accomplished by alternatively placing the experimental group with a lactating dam and a nipple ligated dam while the control group was alternately placed with two lactating dams (Lynch, 1976a). Both techniques result in a quantitative decrease in the amount of milk available to the nursing pups. The large litter technique also results in possible cage crowding with consequent increases in nest temperature and possible changes in maternal behavior; dietary restriction of the lactating dam is likely to result in a change in maternal behavior (Frankovà, 1971, 1972).

Many of the first studies of the effects of early nutritional insult on behavioral development in subhuman animals focused on learning situations involving maze performance in rats. The results of these studies indicated that rats that had been malnourished in early life performed better than control-fed rats when food reward was used in simple maze tests (Andersen and Smith, 1932; Bernhardt, 1936; Griffiths and Senter, 1954) but worse than controls either

when food reward was used in complex mazes (Baird, Widdowson & Cowley, 1971; Cowley and Griesel, 1959, 1963, 1964, 1966) or in water mazes (Barnes, Cunnold, Zimmerman, Simmons, Macleod & Krook, 1966; Bernhardt, 1936; Caldwell and Churchill, 1967; Cowley and Griesel, 1962, cited in Levitsky and Barnes, 1970; Howard and Granoff, 1968) especially those at lower water temperatures. Although it is apparent that the performances of early malnourished rats are different from normals in a variety of maze behaviors, the mechanism of this differential performance is not clear. Alternative interpretations are possible for both the results of studies using dry mazes with food reinforcement and those using water mazes with escape as reinforcement. The responses of malnourished animals to food may account for some of the performance differences in dry mazes. For example, Barnes, Neely, Kwong, Labadan, & Franková (1968) reported greater food spillage, more hoarding, and more huddling around the food cups in systematic observations of animals that were either malnourished at the time of testing, or had previously been malnourished. Others (Blackwell, Blackwal, Yu, Weng, & Chow, 1969; Chow, Blackwell, Blackwal, Hou, Analine, & Sherwin, 1968; Hseuh, Blackwell, & Chow, 1970; Lee and Chow, 1965, 1968) reported that gestationally and/or lactationally malnourished animals consumed more food per unit of body weight than did controls of equal size, weight, or age. In fact, the results of studies in rats (Hseuh et al, 1970) as well as in humans (Chow et al., 1968) suggest that malnourished organisms may have a metabolic derangement that is reflected in poor food

generalize from one strain of rats to another or from one species to another.

In sum, interpretation of the findings of experimental studies in which amount of activity in the open field is used to assess the behavior of nutritionally deprived animals must take into account all of the abovementioned variables in addition to the possible contribution of the animal's size, its weight, and its sensory or motor capabilities.

Open field behaviors other than amount of activity of inadequately nourished animals have also been systematically observed. Simonson et al, (1971) reported that their experimental rats entered the central portions of the open field significantly fewer times than did the control animals and had significantly longer latencies to exit from the "entry square". Franková and Barnes (1968a) reported that 50 day old rats that had been nutritionally restricted only during the lactation period had significantly longer periods of inactivity, whereas rats nutritionally restricted to 49 days of age had longer durations of supported or non-supported vertical rearing, shorter durations of grooming, and shorter durations of inactivity compared to controls. Following 25 and 35 days of nutritional rehabilitation, the relationship between the well- and poorly-nourished rats was reversed on all behaviors. Levitsky and Barnes (1972) reported that following of other animals was lower and fighting with other animals higher in rats that had been malnourished for the first seven weeks of life than in control-fed rats. Finally, many of these

studies observed fecal boluses in the experimental situation. The consistent observation was that there is more defecation in the experimental animals. However, it must be noted that the amount of defecation may reflect the dietary, as well as the developmental history of an animal (Tobach and Schneirla, 1962) and not necessarily its reaction to an unfamiliar environment.

In addition to the earlier-mentioned problems of comparing the results of different studies of open field behavior in early malnourished rats, there are other concommitants of malnutrition that complicate a clear understanding of the results of such studies. Early malnutrition results in a lag of two to three days in certain aspects of development which may result in an organism which is unable to optimally utilize information from the environment. For example, eye lid opening (Cowley and Griesel, 1963; Stephan, Simonson, Hanson & Chow, 1970; Widdowson and McCance, 1960), earflap unfolding (Cowley and Griesel, 1963), response to sound (Cowley and Griesel, 1963), development of homeostatic temperature control (Heggeness, 1962) and development of spontaneous movements (Franková, 1968) all occur at a later age in malnourished than in well-nourished animals. This would most likely affect responsiveness and activity in the open field. Furthermore, Levitsky & Barnes (1970b) have proposed that animals that are currently or have previously been inadequately nourished have a qualitatively different "repertoire of responses" to environmental stimuli than do normal animals and they have argued that as a result of the altered susceptibility to

environmental inputs during early development, the inadequately nourished animal is subsequently unable to behave in a manner characteristic of animals who have evolved normal patterns of responding to environmental stimuli. Another important consideration in assessing the effects of an altered organism-environment interaction is the reciprocally attenuated mother-pup interaction mentioned earlier (Frankovâ 1971, 1972). The various early experiences which are different in early malnourished than in control-fed animals may serve to mediate and/or determine the altered responses in the open field.

Effects of early malnutrition and early handling on behavior.

Although the mechanisms by which open field behavior is altered in malnourished animals is unknown, the finding of a differential response to the open field in malnourished versus normal animals is well-documented. Early malnutrition also affects other related behaviors: Frankovâ (1968) reported that the development of spontaneous activity in rats occurred earlier in intermediate and large sized litters than in either extremely large or small litters. This finding suggested an interaction between intra-litter stimulation and neonatal nutrition. Frankovâ also reported that daily stimulation (weighing, stroking, and marking) during the first four weeks of life resulted in a narrowing of the differences between those animals nursed in small litters and those nursed in large litters in the development of spontaneous exploratory activity and in standing up reactions when observed at 90 and 100 days of age. Considering these experimental findings, Frankovâ (1968) suggested that it may

nutritional deficiency and early handling on Hebb-Williams maze performance have also been reported in several studies. Denenberg and Morton (1962) found that early-handled animals made fewer errors than non-handled rats in a Hebb-Williams maze whereas Cowley and Griesel (1959) reported the opposite for early malnourished animals.

Various experimenters have also reported that early malnutrition and early handling have opposite effects on measures of physical growth. Rats which had been handled in early life were reported to be heavier than non-handled rats (Altman, 1968; Denenberg, 1962; Levine, 1959) and rats fed low protein diets in early life or nursed by dams fed low protein diets were lighter than well-nourished rats (Franková and Barnes, 1968a, 1968b). Brain weights are similarly altered: they are greater in early handled than in non-handled animals (Tapp and Markowitz, 1963), and lower following early nutritional deficiency than following adequate nutrition (Winick and Noble, 1965). Finally, Dobbing (1964) reported that undernutrition in early life in rats resulted in a lesser degree of central nervous system myelination than adequate nutrition, and Levine and Alpert (1959) reported that rats that were handled in early life had more rapid myelination than rats that were not handled in early life.

Since there is no way of determining whether the animals used in nutrition studies in different laboratories were subjected to similar amounts of non-experimental handling, or whether the animals in handling studies from different laboratories were fed in the same manner, it is difficult to draw conclusions regarding the relationship

between the effects of nutritional status and handling in early life on the basis of these ad-hoc comparisons. However, considering some of the apparently opposite effects on behavioral and physiological parameters of early handling and early malnutrition, Frankovà's (1968) suggestion that it may be possible to counteract some of the adverse effects of early malnutrition by simultaneously providing extra handling deserves further study. Since the physiological maturation necessary for the reception and utilization of at least some important environmental inputs may be occurring somewhat later in malnourished than in well nourished animals, it is reasonable to argue that the provision of external stimulation which may 1) accelerate physiological development, 2) act on a common hormonal system, or 3) make up some of the deficit in certain kinds of external stimulation suffered by malnourished animals, could compensate, at least in part, for the effects of early malnutrition.

There have been only two experiments which directly examined the effects of simultaneous early handling and early malnutrition on behavioral and physiological development in rats. The first of these was conducted by Frankovà (1968), who raised rats in litters of 4, 9, 13, and 17, with half of each group being weighed, stroked, and manipulated while undergoing daily marking, and the other half not being experimentally stimulated. At 90 days of age, horizontal and vertical activity in the open field was greater for the handled than for the non-handled animals of each litter size, with the greatest effect of handling on those animals from the litters of 9 and 13 pups. An inverted U-shaped function for the effects of early stimulation on later open field behavior was suggested since handling had a considerable effect

on the later behaviors of those animals reared with moderate dietary inadequacy but very little effect on the later behaviors of those animals which were either most well-nourished or least well-nourished.

Following Frankova's initial demonstration of a relationship between early handling and early malnutrition, Levitsky and Barnes (1972) systematically studied the effects of early nutritional deprivation and of simultaneous variations in environmental stimulation on open field behavior. The rat pups either nursed from dams fed low protein diets and were themselves fed low protein diets for four weeks postweaning, or they nursed from dams fed high protein diets and were themselves fed high protein diets for four weeks postweaning. Animals from each of these two groups were also assigned to one of three different environmental conditions: 1) tactile stimulation[1] preweaning and postweaning, and "complex environment"[2] for four weeks postweaning; 2) no tactile stimulation preweaning, and laboratory cages postweaning; or 3) no tactile stimulation preweaning, and isolation cages for four weeks postweaning. After ten weeks of control feeding and laboratory cage living for all animals, they were tested for locomotion, following, mutual grooming, and fighting in the open field as well as entry into a partition adjacent to the chamber. The

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1. Tactile stimulation consisted of handling the pups for approximately three minutes every day.

2. The "complex environment" was a 58 cm x 34 cm wooden box equipped with objects which the rats could manipulate. They were placed in this box with five other animals for one hour periods five days a week.

early isolated animals exhibited significantly greater locomotion than the control or stimulated animals, with isolation having a greater effect on those rats that had been malnourished than on those rats that had been adequately nourished. With respect to fighting and following behaviors, Levitsky and Barnes found that those rats that had been well-nourished responded the same regardless of their early rearing condition while those rats that had been isolated and malnourished entered the partition adjacent to the chamber significantly fewer times than did any of the well nourished animals, or the animals that had been stimulated simultaneously with their malnutrition. These results indicate that early rearing conditions have a profound influence on an animal's long term response to early malnutrition.

The present study is an extension of the Levitsky and Barnes (1972) study of the relationship between early nutritional status and early handling on open field behavior in rats. The main purpose of the present experiment was to examine the effects of these two events on changes in open field behavior during the course of development from early post-weaning life until puberty and also in adulthood. The study is specifically addressed to the question of how the simultaneous provision of daily handling and dietary manipulation for the first three weeks of postnatal life affects activity in the open field over the course of development until puberty. While the Levitsky and Barnes study (1972) represents the initial step in determining the long term effects of concurrent malnutrition

and handling, the present experiment is an attempt to answer some of the numerous questions which remain. First, the "environmentally stimulated" animals in the Levitsky and Barnes study were handled during the first seven weeks of life as well as being provided with "complex environments" one hour a day from four through seven weeks of age. It cannot be determined from this design whether the effects that their "environmental stimulation" had on the open field behavior of malnourished animals were due to one or the other of the multiple stimuli provided, or whether they were due to the seven week period of handling. The present study sought to determine whether only one external stimulus (handling) during a more limited period of time (the nursing period) would differentially effect the developmental course of open field behaviors of malnourished and well-nourished rats. Second, while the Levitsky and Barnes study allowed ten weeks of nutritional rehabilitation before behavioral testing began, the present experiment attempted to determine the nature and direction of the behavioral differences among the groups of animals during the course of development from weaning to 50 days of age as well as following 10 weeks of nutritional rehabilitation. Third, while Levitsky and Barnes measured only horizontal activity in the open field, the present study differentiated between horizontal activity in the peripheral squares and that in the central squares in order to determine whether some groups of animals would preferentially engage in locomotor activity in certain parts of the open field. Vertical activity with the support of the walls of the

open field and that without support of the walls of the open field was also measured --- both as another measure of activity and also to determine whether physical strength was an important contributor to open field activity of malnourished animals. Attention to a novel object in the open field was also examined in the present experiment to determine whether the different groups of animals would approach or avoid unfamiliar stimuli. Finally, the present study sought to determine the relationship between behavior in the open field and the size and number of brain cells, and body, brain, liver, and adrenal gland weights.

The present investigation is therefore a longitudinal study of the effects of both early malnutrition and early handling on ontogenetic changes in open field behavior in rats. The study was designed to determine: 1) the effects of malnutrition during the lactation period on behavior in the open field; 2) the effects of handling during the lactation period on the same behavior; 3) the relationship between early nutritional status and early handling in determining such behaviors and 4) the nucleic acid responses in the brains of malnourished and well-nourished and handled and non-handled animals following five weeks of dietary rehabilitation.

METHODOLOGY

Subjects

The subjects were 128 offspring (80 males and 48 females) of 16 Sprague-Dawley dams obtained from Carworth on the fourth gestational

day. The dams were maintained in the laboratory on Purina rat chow until parturition.

Laboratory Conditions

The gestational dams were caged individually in 10" x 16" transparent plastic cages with ample wood shavings for nesting material. The same cages were used throughout the experiment. They were kept in a small room in which air circulation was maintained. The temperature was kept constant at 72°F. An automatic timer permitted alternating 12 hour periods of light and dark, so that the room was lit from 7:00 AM to 7:00 PM, and unlit from 7:00 PM to 7:00 AM.

At 21 days of age, all pups were weaned, ear punched for identification, and assigned to cages with two to four same-sexed mates belonging to the same experimental group.

Experimental Design

All pups born within a 12 hour period were pooled and randomly assigned to one of four experimental groups: Handled malnourished (HM), non-handled malnourished (NM), handled well-nourished (HW), and non-handled well nourished (NW). There were 32 pups in each group.

Those pups assigned to the two well-nourished groups (HW, NW) were fostered to dams fed a 25% protein diet, while those pups assigned to the two malnourished groups (HM, NM) were fostered to dams fed a 12% protein diet. Half of the pups in each of the two nutrition groups was assigned to one of the two handled conditions

(HM, HW) and were handled daily from birth to weaning at 21 days of age. The remaining half of the pups in each of the two nutrition groups was assigned to one of the two non-handled conditions (NM, NW). The non-handled pups were not disturbed during the nursing period except for one cage cleaning.

Dietary Manipulation Procedure

Preparturient dams were fed Purina lab chow ad lib (approximately 18% protein).

Dietary manipulation of the pups during the nursing period took place via the maternal dietary intake. The pups of the two malnourished groups (HM, NM) were nursed by dams receiving a diet consisting of 12% casein from within 12 hours after parturition until weaning. This results in a decline in the milk output within three days (Meuller and Cox, 1945; Venkatachalam and Ram, 1964). The pups of the two well-nourished groups (HW, NW) were nursed by a dam receiving a diet of 25% casein during this period. (See Table 1 for the nutrient content of the diets.) The components of the diets were combined in the prescribed proportions by the experimenter, and mechanically blended until homogenous.

Following weaning, all pups were fed Purina Lab Chow ad libitum.

Handling Procedure

The pups in the two handled groups (HM, HW) were individually handled each day for the first 21 days of postnatal life. The pups in the two non-handled groups (NM, NW) were not experimentally handled during this period. The handled pups were removed from

Table 1. Nutrient Content of Diets

Nutrient	Malnourished (%)	Well Nourished (%)
Casein	12.0	25.0
Glucose monohydrate[1]	65.7	52.7
Hydrogenated vegetable oil[2]	15.0	15.0
Mineral salt mixture[3]	4.0	4.0
Choline Dihydrogen citrate	0.3	0.3
B Vitamins[4]	2.0	2.0
Fat soluble vitamins[5]	1.0	1.0
Total	100.0	100.0

1. Cerelose, Corn Products Company, Argo, Illinois
2. Primex, Procter and Gamble Company, N. Y.
3. Mineral Salt mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio
4. B Vitamins in 2.0 g Glucose monohydrate, in the following quantities:

Thiamin HCL	0.40 mg
Riboflavin	0.80 mg
Pyridoxamine	0.40 mg
CaPantothenate	4.00 mg
Niacin	4.00 mg
Inositol	20.00 mg
Biotin	0.02 mg
Folic Acid	0.20 mg
Vitamin B_{12}	0.03 mg
Menadione	1.00 mg

5. Fat soluble vitamins in 1.0 g corn oil in the following quantities:

Vitamin A Acetate	0.31 mg
Vitamin D (Calciferol)	0.0045 mg
Vitamin E (Alpha tocopherol)	5.00 mg

their cages each morning by the experimenter who was wearing rubber gloves. Each pup was placed in a large metal container with three other pups and was in the container for 20 minutes. At randomly determined times over the 20 minute period, one of the four pups was individually removed from the container, held in the experimenter's hand, and vigorously stroked on its back with the experimenter's thumb for two minutes. The pup was then returned to the container.

Behavioral Testing Apparatus

The open field apparatus consisted of a 4' x 4' x 18" chamber. Three walls were plywood painted black, and one wall was plexiglass to permit eye-level observation. There was no ceiling. The floor was marked off into 25 equal-sized squares with 3/8" plastic tape. A red rubber ball, 2½" in diameter was suspended from one corner by a rubber band to within 2" of a corner square ("novel square") (Figure 1).

Behavioral Testing Procedure

Half of the animals in each of the four groups (n = 16 from each group, total = 64) was tested in the open field at 22, 23, 29, 36, and 50 days of age (early tested).[1] The other half of each

1. The entire experimental procedure was carried out twice: First in January, February and March of 1972, and again in April, May and June of 1972. This was done so that all animals could be observed in the open field during a four hour period. After reviewing the initial results it was decided to test the animals one more time when the testing was repeated. Therefore, all 16 animals in each experimental group were tested at 22, 23, 29, and 36 days of age, but only eight animals were tested at 50 days of age.

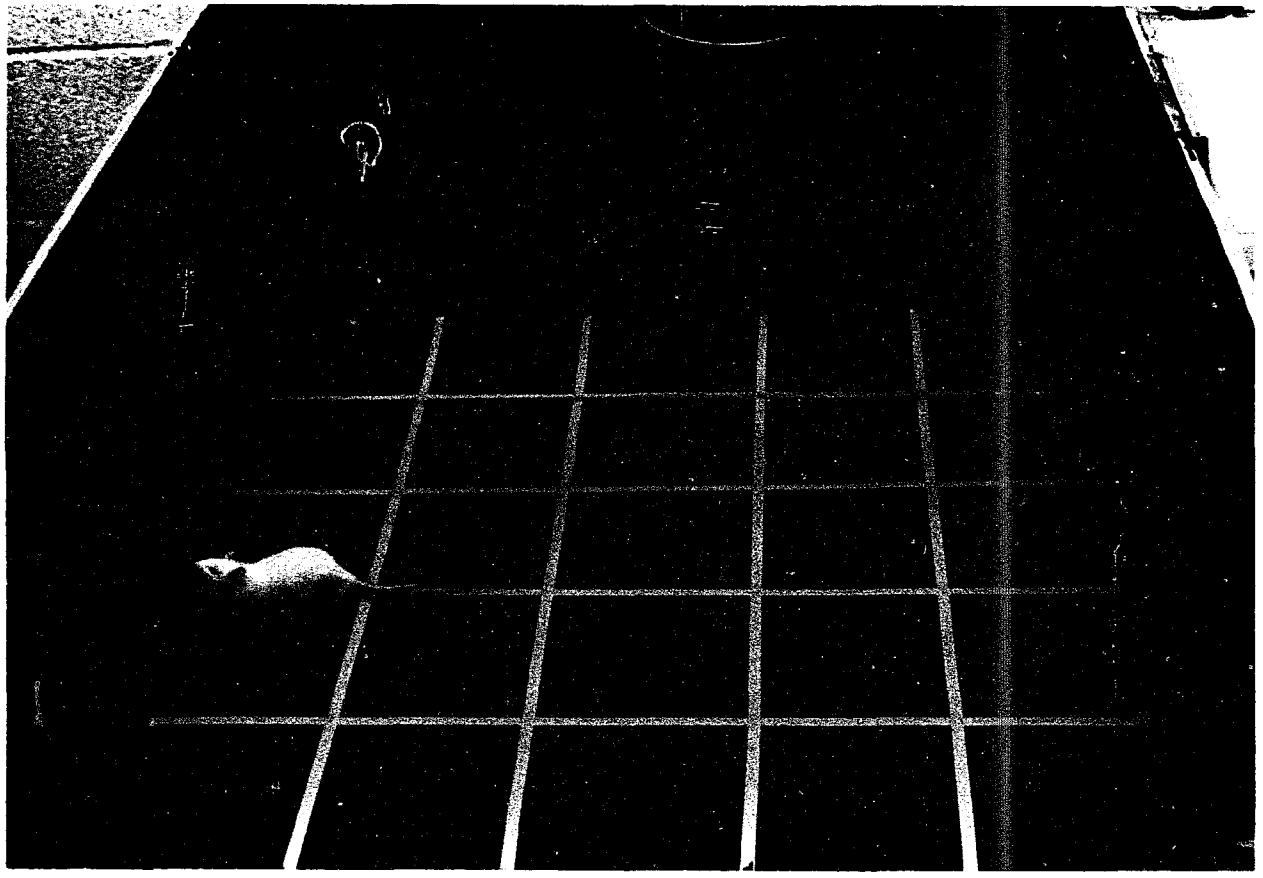

FIGURE 1. The Open Field

group (n = 16 from each group, total = 64) was first tested at 90 days of age and retested at 91 days of age (later tested).[1]

Behavioral testing was always carried out between 8:00 AM and 12:00 noon. Each animal was identified by a number during testing so that the experimenter was blind with respect to whether the animal being tested had been handled. However, the difference in size of the malnourished and well nourished animals made their identity difficult to conceal. Each animal was dark adapted for 30 minutes prior to testing and testing was carried out in a small room, lit by only a red 60 watt lamp. Each animal was removed from his cage by sliding cardboard underneath and an empty two-pound coffee container over his head. The subject was carried to the testing chamber in this container to avoid handling. The animal was placed in the chamber by inverting the container in the corner opposite the novel square. Testing began when the animal's feet met the chamber floor, and continued for 20 minutes. Observations were continuously made by the experimenter with the use of a manual

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1. It was originally intended that one group of animals would be observed in the open field at 22 and 23 days of age and that the other group would be observed at 90 and 91 days of age. These ages were chosen so that comparisons could be made between open field behavior immediately following the experimental manipulations and following 10 weeks of dietary rehabilitation. After the initial observations at 22 and 23 days of age, however, the experimenter decided to continue testing these animals at weekly intervals. Thus, what was originally intended as a cross-sectional study became a longitudinal study. Unfortunately, the laboratory in which the study was performed was moved, and since it was not possible to reproduce the housing and other conditions, crossectional controls for the different ages of testing could not be undertaken.

five-channel counter using one channel for each of five measures, and a timer to record the sixth measure with a stop watch. The following behaviors were recorded: 1) The number of times the 16 peripheral squares were entered. An entrance was scored when both the front and the hind legs had crossed the taped line bordering the square, 2) The number of times the nine central squares were entered, 3) The number of vertical rears supported by the chamber walls. A response was considered a "rear" when the front legs had been brought up above the level of the rear hips, 4) The number of vertical rears unsupported by the chamber walls, 5) The number of times the "novel square" was entered, and 6) The time, in seconds, recorded on a stop watch, spent pawing or chewing the novel object.

All fecal boluses were removed and the apparatus was cleaned with a wet paper towel following each 20 minute test session. The number of fecal boluses were not documented for study because in preliminary observations it was found that these rats rarely defecated in the open field apparatus. In addition, differential defecation rates may have been due to the different nutritional histories of the animals in this study, and not to the animals' reactions to unfamiliarity.

Observations were also made of the patterns of activity that the animals displayed and the experimenter noted qualitative aspects of behavior such as "rapid bursts of activity" or "steady pace of activity" for each animal. Whether the animals displayed periods

of inactivity and in which parts of the apparatus these behaviors occurred was also noted.

Somatometric Measurements and Chemical Testing Procedure

Within 12 hours of parturition, all dams, as well as the eight pups assigned to each dam were weighed. Weights were obtained again for the dams and the pups individually on the day of weaning, and weekly thereafter.

The 64 animals that had been behaviorally tested in the open field from 22 to 50 days of age were killed at 55 days of age. As was the case for the behavioral testing procedure, the experimenter was blind only with respect to whether the animal being killed had been handled. The brains, livers, and adrenal glands were removed. Wet weights were obtained for forebrain, midbrain, and cerebellum, as well as for livers and adrenals. Liver and adrenal gland weights were taken in order to determine whether there were any differences among the four groups in non-CNS organs. The forebrains were homogenized immediately upon removal in 10% homogenates of 0.2 M sucrose in TKM buffer, and stored at -20°C until chemical analysis was carried out the following day. Chemical analysis consisted of extraction of RNA, DNA, and protein by the methods of Munro and Fleck (1966) and was carried out in test tubes that were labeled according to experimental group. (See Appendix 1 for assay procedure.) Quantification of extractions were measured by UV absorption in a Beckman DU spectrophotometer at 260 Å for RNA, at 269 Å for DNA and at 540 Å for protein prepared by the biuret method. All chemical

procedures were carried out by the experimenter and precisely followed those used by Winick and Noble (1965, 1966) for the extraction of nucleic acids.

Statistical Analysis

A 2 x 2 analysis of variance (ANOVAR) was performed on the data for each of the six open field measures and each of the chemical measures. Whenever the over-all level of significance for either of the main effects (nutrition and handling) or their interaction exceeded $p < 0.05$, a post hoc analysis of the data was performed (Hays, 1963, Pp. 485-487) to permit separate comparisons among the four groups. The data were not analyzed for sex differences because the number of females in each group was too small (n = 6).

To assess the changes in the open field activity with change in age (from 22 to 36 days of age)[1], a repeated measures ANOVAR was performed for each of the six behavioral measures.

RESULTS

Open Field Behavior

Horizontal Activity in Peripheral Squares

Animals that had been handled during the nursing period (HW and HM) were more active in the peripheral squares of the open field than animals that had not been handled during this period (NW and NM).

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1. Activity from 22 to 36 days of age was used in this analysis instead of activity from 22 to 50 days of age because the same 16 animals in each group were tested at 22, 23, 29, and 36 days of age, while only eight of these animals were tested at 50 days of age.

As can be seen in Table 2a and Figure 2, at 22, 23, 29, 36 and 50 days of age, animals that had been handled during early life entered more peripheral squares than animals that had not been handled regardless of whether they had been nursed by malnourished or well-nourished dams. The greater activity of the handled rats over the non-handled rats in the peripheral squares of the open field was statistically significant at all ages from 22 to 50 days of age (Table 2b).

Animals that had been handled and nursed by dams fed high protein diets (HW) were more active in the peripheral squares of the open field from 22 to 50 days of age than were animals that had been nursed by dams fed high protein diets but had not been handled (NW). The greater activity of the early well-nourished animals over the malnourished animals without regard to early handling was statistically significant only at 22 days of age (Table 2b).

As can be seen in Table 2a and Figure 2, the HW animals entered more peripheral squares than each of the other three groups at each age (to 50 days of age). In fact, the HW animals entered significantly more peripheral squares than their malnourished counterparts at 22 days of age as well as significantly more peripheral squares than their non-handled counterparts at both 22 and 23 days of age (Table 2c), resulting in a statistically significant interaction between handling and nutrition on these two days (Table 2b).

As can be seen in Figure 2, there was a general increase in peripheral square activity with increase in age in all four groups. There was, however, a statistically significant interaction between

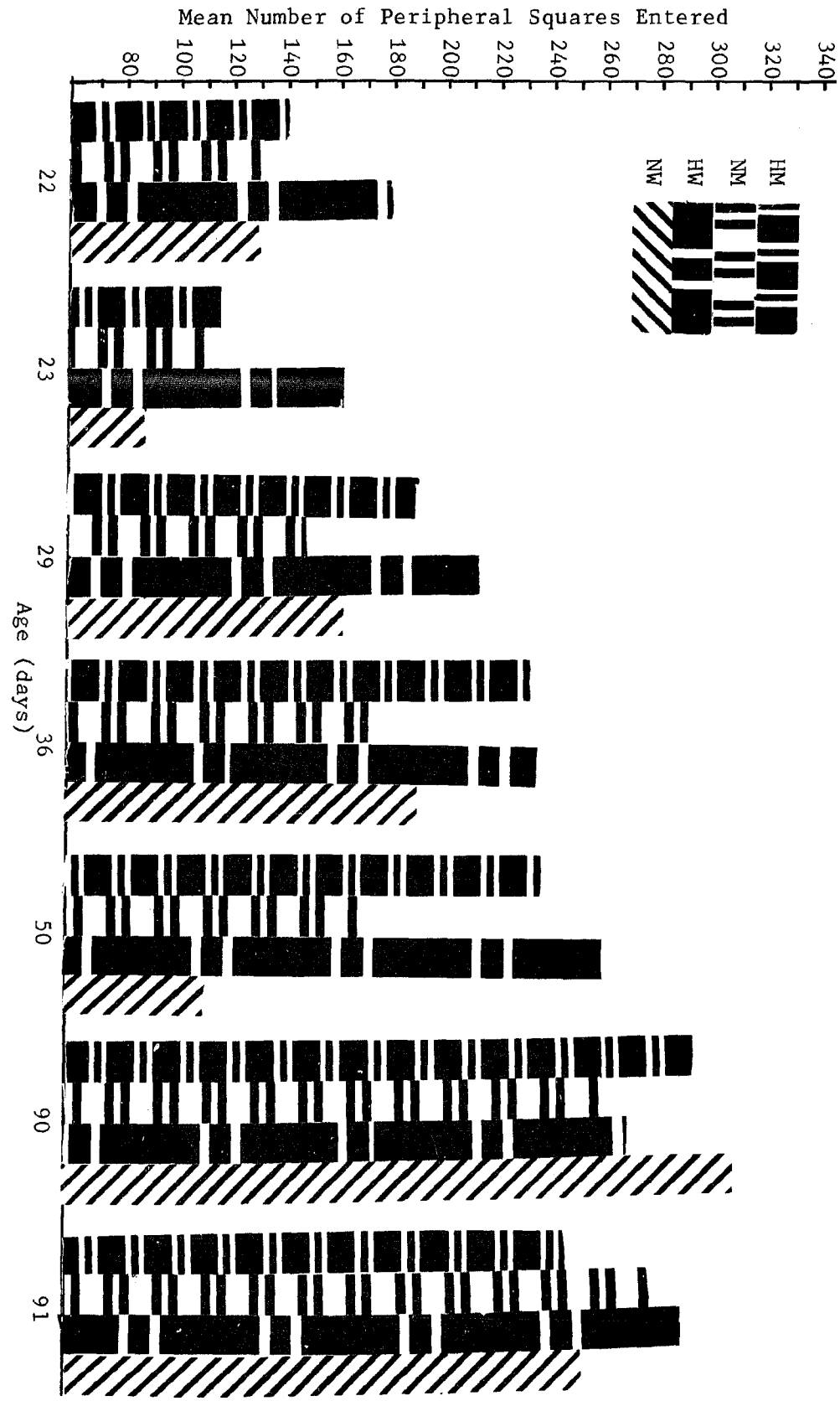

FIGURE 2

Number of Peripheral Squares Entered

Table 2a

Number of Peripheral Squares Entered

Age (days)		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
22	$\bar{X}$	140.4	129.4	179.7	128.3
	s.d.	41.3	42.8	32.8	33.8
23	$\bar{X}$	117.5	110.4	161.8	84.8
	s.d.	38.9	46.3	31.8	34.1
29	$\bar{X}$	189.2	145.3	216.2	162.5
	s.d.	39.8	72.0	58.3	70.5
36	$\bar{X}$	234.4	171.1	239.5	189.4
	s.d.	55.6	52.8	55.6	51.6
50	$\bar{X}$	239.6	172.0	258.8	108.0
	s.d.	28.3	93.8	45.4	89.3
90	$\bar{X}$	289.6	258.5	270.3	310.1
	s.d.	51.5	111.0	56.3	225.0
91	$\bar{X}$	245.1	273.5	290.1	251.0
	s.d.	84.1	103.9	72.1	97.7

Table 2b

ANOVAR for Peripheral Squares Entered

Age (days)	Source of Variation	d.f.	Sums of Squares	Mean Square	F	P
22	Handling	1	15,562.56	15,562.56	10.83	<0.005
	Nutrition	1	5,814.06	5,814.06	4.04	<0.05
	Interact.	1	6,561.00	6,561.00	4.56	<0.05
	Error	60	86,168.13	1,436.13		
23	Handling	1	28,350.14	28,350.14	10.49	<0.05
	Nutrition	1	1,396.89	1,396.89	0.51	NS
	Interact.	1	19,565.02	19,565.02	7.24	<0.01
	Error	60	162,057.19	2,700.95		
29	Handling	1	35,672.82	35,672.82	9.42	<0.05
	Nutrition	1	7,326.15	7,326.15	1.93	NS
	Interact.	1	360.15	360.15	0.09	NS
	Error	60	227,022.60	3,783.71		
36	Handling	1	48,223.35	48,223.35	9.20	<0.005
	Nutrition	1	2,053.35	2,053.35	0.39	NS
	Interact.	1	660.02	660.02	0.12	NS
	Error	60	314,489.40	5,241.49		
50	Handling	1	94,613.00	94,613.00	19.26	<0.001
	Nutrition	1	4,186.10	4,186.10	0.85	NS
	Interact.	1	14,112.00	14,112.00	2.87	NS
	Error	28	137,530.00	4,911.70		
90	Handling	1	0.26	0.26	0.00	NS
	Nutrition	1	1,928.80	1,928.80	0.10	NS
	Interact.	1	22,817.00	22,817.00	1.23	NS
	Error	60	110,688.00	18,448.00		
91	Handling	1	344.54	344.54	0.04	NS
	Nutrition	1	1,525.50	1,525.50	0.19	NS
	Interact.	1	13,689.00	13,689.00	1.74	NS
	Error	60	469,920.00	7,832.00		

age, nutrition and handling ($F = 2.78$; $df = 3, 54$; $p < 0.05$). This may have resulted from the relatively greater increase in peripheral square activity from 22 to 36 days of age in the HM animals than in either their non-handled counterparts (NM) or in their well-nourished

Table 2c

Post Hoc Comparisons for Peripheral Squares Entered (p Values)

	Age (days)						
	22	23	29	36	50	90	91
HW vs NW	< 0.005	< 0.005	NS	NS	NS	NS	NS
NM vs NW	NS	NS	NS	NS	NS	NS	NS
HM vs NM	NS	NS	NS	NS	NS	NS	NS
HW vs HM	< 0.05	NS	NS	NS	NS	NS	NS

counterparts (HW). It may also have resulted from the relatively lesser increase in peripheral square activity from 22 to 36 days of age in the NM animals than in either their well-nourished counterparts (NW) or their handled counterparts (HM).

There were no systematic differences in the number of peripheral squares entered among the four groups of animals tested for the first time at 90 days of age and retested at 91 days of age (Table 2b).

To determine whether the differences among the groups of younger animals for the 20 minute testing sessions were a function of differences in initial performance in the open field, or whether they were

due to differences which were maintained throughout the entire testing session, the responses made during the first five minute segment were analyzed. The mean number of peripheral squares entered during the first five minutes from 22 to 50 days of age were as follows[1]:

Day of Age	HM	NM	HW	NW
22	61.0	46.5	86.3	64.6
23	53.9	46.1	71.2	41.1
29	73.0	69.9	104.4	67.8
36	91.9	76.3	112.1	83.3
50	110.6	78.3	105.0	61.0

The handled animals were significantly more active in the peripheral squares than the non-handled animals during the first five minute interval at all ages observed. ($F = 8.04$; $df = 1, 60$; $p < 0.01$ at 22 days of age; $F = 9.42$; $df = 1, 60$; $p < 0.005$ at 23 days of age; $F = 5.69$; $df = 1, 60$; $p < 0.025$ at 29 days of age; $F = 8.27$; $df = 1, 60$; $p < 0.01$ at 36 days of age; $F = 13.44$; $df = 1, 28$; $p < 0.001$ at 50 days of age). The well-nourished animals entered significantly more peripheral squares than the poorly nourished animals during the first five minutes only when tested at 22 days of age ($F = 11.51$; $df = 1, 60$; $p < 0.005$). These results indicate that the statistically significant differences in

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1. Data from the first five minute segment of the testing sessions on days 90 and 91 were not included here or in subsequent sections (for other open field measures) because preliminary analysis failed to reveal any differences between the results obtained in early-tested animals and those obtained in adult animals.

peripheral square activity among the four groups for the first five minutes of testing were the same as those for the entire 20 minute testing period.

Summary. When tested immediately following weaning, the consistent finding for horizontal activity in the peripheral squares was that those animals that had been handled in early life entered more squares than those animals that had not been handled in early life. When the two groups of animals nursed by well-nourished dams were compared to the two groups of animals nursed by malnourished dams, there were no significant differences in the number of peripheral squares entered except at 22 days of age. The HW animals were consistently more active in the peripheral squares than any of the three other groups.

When tested in adulthood, there were no consistent differences among the four groups of animals on the number of peripheral squares entered.

Horizontal Activity in Central Squares

As was the case for peripheral square activity, the early handled animals (HW and HM) entered more central squares at each age from 22 to 91 days than the non-handled animals (NW and NM) regardless of whether they had been nursed by high or low protein dams (Table 3a and Figure 3). The greater central square activity of the early handled rats over the non-handled rats was statistically significant at all ages for both the younger animals (to 50 days of age) and the animals tested in adulthood (91 days of age) (Table 3b). Whereas

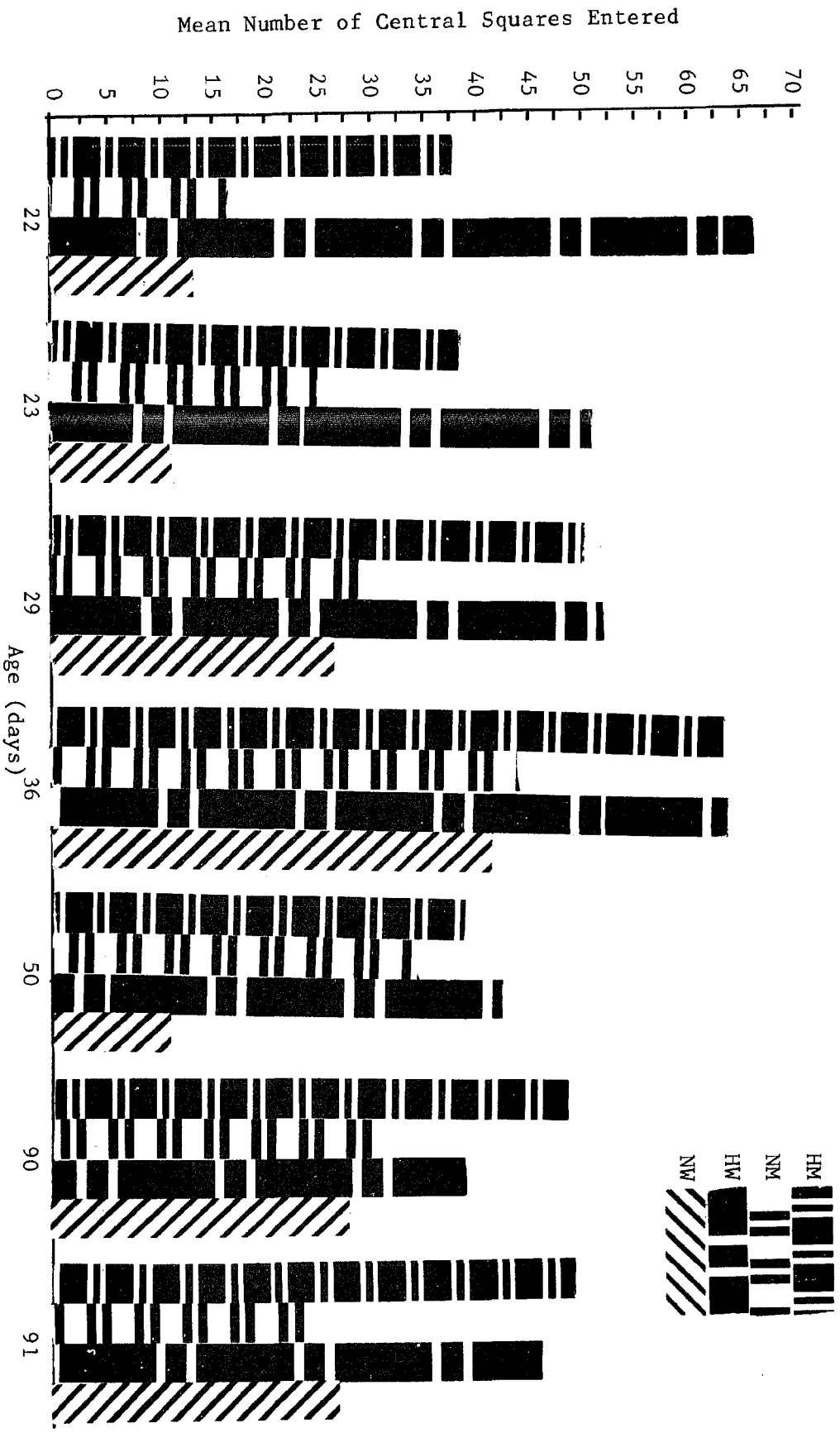

FIGURE 3

Number of Central Squares Entered

Table 3a

Number of Central Squares Entered

Age (days)		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
22	$\bar{X}$	37.9	16.8	66.5	13.1
	s.d.	24.0	8.4	25.7	12.0
23	$\bar{X}$	38.5	25.3	51.4	11.2
	s.d.	17.8	19.0	17.4	14.4
29	$\bar{X}$	50.5	29.6	52.5	27.0
	s.d.	4.8	24.8	17.9	23.0
36	$\bar{X}$	63.9	43.7	64.5	42.1
	s.d.	20.1	34.6	21.2	29.2
50	$\bar{X}$	40.0	34.6	43.8	11.9
	s.d.	8.2	27.0	14.7	16.5
90	$\bar{X}$	49.9	32.4	39.0	29.0
	s.d.	33.5	30.9	24.7	25.0
91	$\bar{X}$	50.8	25.6	47.8	27.5
	s.d.	35.0	24.0	33.4	15.1

Table 3b

ANOVAR for Central Squares Entered

Age (days)	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
22	Handling	1	22,238.26	22,238.26	61.29	<0.001
	Nutrition	1	2,487.51	2,487.51	6.85	<0.025
	Interact.	1	4,176.40	4,176.40	11.51	<0.001
	Error	60	21,769.69	362.82		
23	Handling	1	11,395.56	11,395.56	37.91	<0.001
	Nutrition	1	6.25	6.25	0.00	NS
	Interact.	1	2,916.00	2,916.00	9.70	<0.005
	Error	60	18,033.63	300.00		
29	Handling	1	8,073.60	8,073.60	16.38	<0.001
	Nutrition	1	1.66	1.66	0.00	NS
	Interact.	1	77.07	77.07	0.15	NS
	Error	60	29,563.80	492.73		
36	Handling	1	8,640.00	8,640.00	11.89	<0.001
	Nutrition	1	153.60	153.60	0.21	NS
	Interact.	1	38.40	38.40	0.05	NS
	Error	60	43,569.00	726.15		
50	Handling	1	2,775.10	2,775.10	8.61	<0.01
	Nutrition	1	722.00	722.00	2.24	NS
	Interact.	1	1,404.50	1,404.50	4.36	<0.05
	Error	28	9,018.30	322.08		
90	Handling	1	2,714.90	2,714.90	3.38	NS
	Nutrition	1	728.27	728.27	0.90	NS
	Interact.	1	204.27	204.27	0.25	NS
	Error	60	48,144.60	802.41		
91	Handling	1	6,261.80	6,261.80	7.58	<0.01
	Nutrition	1	3.68	3.68	0.00	NS
	Interact.	1	72.94	72.94	0.08	NS
	Error	60	49,503.60	825.06		

the HW animals entered a significantly greater number of central squares than the NW animals at 22, 23, 29, and 50 days of age, the significantly greater number of central squares entered by the HM animals over the NM animals did not persist beyond 22 days of age (Table 3c).

As can be seen in Figure 3, the number of central squares entered by the HM, NM, and NW groups increased from 22 to 36 days of age, and decreased at 50 days of age. However, the amount of change

Table 3c

Post Hoc Comparisons for Central Squares Entered

	Age (days)						
	22	23	29	36	50	90	91
HW vs NW	<0.001	<0.001	<0.05	NS	<0.025	NS	NS
NM vs NW	NS	NS	NS	NS	NS	NS	NS
HM vs NM	<0.05	NS	NS	NS	NS	NS	NS
HW vs HM	<0.001	NS	NS	NS	NS	NS	NS

in central square activity from 22 to 36 days was not parallel in the four groups --- there was a significant interaction between age, nutrition and handling ($F = 4.08$; $df = 3, 54$; $p < 0.01$). This probably resulted from the decline in central square activity by the HW animals from 22 to 36 days of age and the increase in this activity by the HM, NM and NW animals.

The nature and direction of the group differences in the central square activity of those animals tested for the first time at 90 days of age was similar in all respects to the differences found for the younger animals. At both 90 and 91 days of age, those animals that had been handled during the nursing period entered more central squares than those that had not been handled (Table 3a) and the greater activity of the handled over the non-handled animals was statistically significant at 91 days of age (Table 3b). Individual comparisons among the four groups did not reveal any statistically significant differences at either 90 or 91 days of age (Table 3c).

The number of central squares entered during the first five minute interval of the 20 minute testing sessions were as follows:

Day of age	HM	NM	HW	NW
22	14.0	4.9	26.1	4.5
23	14.1	7.4	21.0	2.6
29	12.8	11.5	18.3	5.3
36	25.4	13.8	26.3	15.0
50	13.1	14.0	14.4	8.0

Similar to the findings for peripheral square activity, the two groups of handled animals entered more central squares than the two groups of non-handled animals during the first five minutes at most ages. The greater central square activity of the handled over the non-handled animals during this interval was statistically significant from 22 to 36 days of age ($F = 34.25$; $df = 1, 60$; $p < 0.001$ for 22 days of age; $F = 45.07$; $df = 1, 60$; $p < 0.001$ for 23 days of age; $F = 8.95$; $df = 1, 60$; $p < 0.005$ for 29 days of age; $F = 17.47$;

df = 1, 60; $p < 0.005$ for 36 days of age). The animals that had nursed from a dam fed a high protein diet entered significantly more central squares than those nursed from a dam fed a low protein diet during the first five minute interval only at 22 days of age ($F = 4.93$; df = 1, 60; $p < 0.05$). As was the case for peripheral square activity, the statistically significant differences among the four groups for the first five minutes of testing were the same as for the 20 minute testing session.

Summary. In general, the picture that emerges from analysis of the data for activity in the central squares is similar to that for activity in the peripheral squares. The early-handled animals were more active in the central squares from 22 to 50 days of age. With the exception of a reversal between the relative positions of the two handled groups of animals (HW and HM) at 36 days of age, the relationships among the number of central squares entered by each of the four groups of animals that was established on the first two days of post-weaning testing was maintained throughout the sequential testing of those animals tested in early life, as well as for those animals first tested at 90 days of age. Whereas the two groups of animals that had nursed from low protein dams (HM and NM) entered intermediate numbers of central squares at all ages except at 36 days, the HW animals entered significantly more central squares than the NW animals at these same ages.

Vertical Activity Supported by Chamber Walls (i.e., vertical activity in peripheral squares).

The number of supported vertical rears made by each of the four groups of animals is presented in Table 4a and in Figure 4. At all ages, the handled animals of both nutritional groups reared more often than their non-handled counterparts and this difference between the handled and non-handled groups was statistically significant at all ages except 91 days (Table 4b). In tests of the younger animals, since supported vertical rearing was greatest in the HW animals, and, except at 29 and 36 days of age, least in the NW animals, there were no statistically significant differences between the well-nourished and the malnourished animals at any age. However, the difference between the HW and the NW animals was statistically significant at 22, 23, 29, and 36 days of age (Table 4c). This difference between the number of supported vertical rears exhibited by the two well nourished groups of animals contributed to the statistically significant interaction between handling and nutrition at 22 and 23 days of age (Table 4b). In tests of the adult animals, supported vertical rearing was greatest among the HM animals and least among the NM animals. There was a statistically significant interaction between nutrition and handling at 90 days of age (Table 4b), which is attributable to the significantly greater number of supported rears made by the HM animals than by the NM animals (Table 4c).

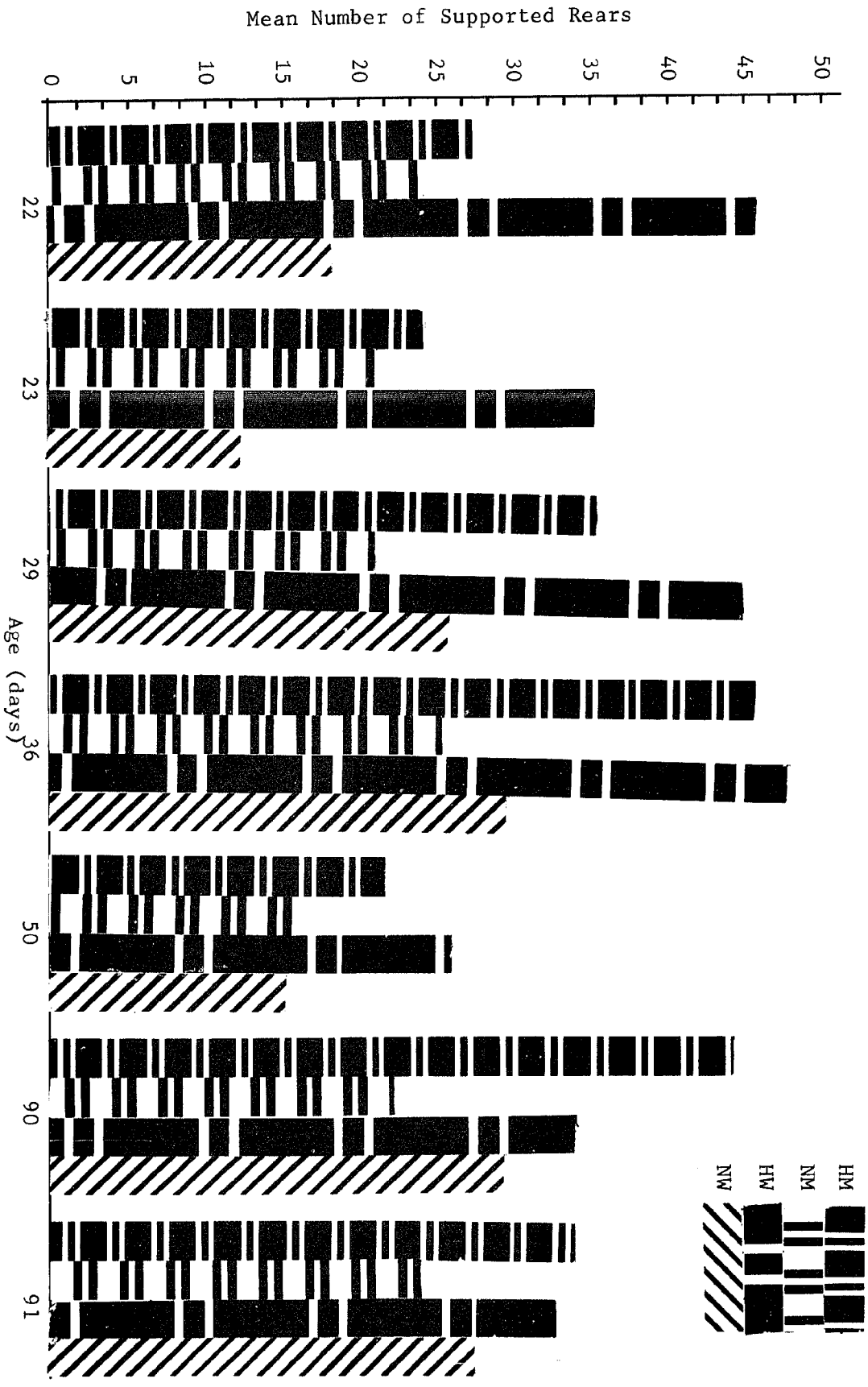

FIGURE 4

Number of Supported Vertical Rears

Table 4a

Number of Supported Vertical Rears

Age (days)		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
22	$\bar{X}$	27.2	23.8	45.6	18.3
	s.d.	13.9	18.4	15.6	6.0
23	$\bar{X}$	24.5	21.4	35.9	12.5
	s.d.	16.7	19.9	14.1	8.1
29	$\bar{X}$	36.1	21.5	45.3	25.7
	s.d.	10.3	14.5	13.9	15.2
36	$\bar{X}$	46.0	25.3	48.0	29.5
	s.d.	14.4	17.2	12.2	16.1
50	$\bar{X}$	22.1	16.1	26.4	15.1
	s.d.	5.1	10.3	8.5	12.1
90	$\bar{X}$	44.9	22.1	33.8	29.0
	s.d.	17.3	14.7	13.1	18.6
91	$\bar{X}$	33.8	24.0	32.1	28.2
	s.d.	12.0	15.0	11.6	13.2

Table 4b

ANOVAR for Supported Vertical Rears

Age (days)	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
22	Handling	1	3,751.56	3,751.56	18.48	<0.001
	Nutrition	1	663.06	663.06	3.26	NS
	Interact.	1	2,280.07	2,280.07	11.23	<0.005
	Error	60	12,176.25	202.93		
23	Handling	1	2,809.00	2,809.00	11.40	<0.005
	Nutrition	1	25.00	25.00	0.10	NS
	Interact.	1	1,640.25	1,640.25	6.66	<0.025
	Error	60	14,775.50	246.25		
29	Handling	1	4,403.27	4,403.27	23.39	<0.001
	Nutrition	1	666.67	666.67	3.54	NS
	Interact.	1	91.26	91.26	0.48	NS
	Error	60	11,294.40	188.24		
36	Handling	1	5,762.40	5,762.40	25.29	<0.001
	Nutrition	1	147.26	147.26	0.64	NS
	Interact.	1	19.27	19.27	0.08	NS
	Error	60	13,667.40	227.79		
50	Handling	1	595.13	595.13	6.75	<0.025
	Nutrition	1	21.13	21.13	0.23	NS
	Interact.	1	55.13	55.13	0.62	NS
	Error	28	2,468.50	88.16		
90	Handling	1	2,721.90	2,721.90	10.61	<0.005
	Nutrition	1	64.20	64.20	0.25	NS
	Interact.	1	1,170.40	1,170.40	4.56	<0.05
	Error	60	15,391.80	256.53		
91	Handling	1	570.89	570.89	3.48	NS
	Nutrition	1	19.63	19.63	0.11	NS
	Interact.	1	101.90	101.90	0.62	NS
	Error	60	9,831.60	163.86		

Table 4c

Post Hoc Comparisons for Supported Vertical Rears (p values)

	Age (days)						
	22	23	29	36	50	90	91
HW vs NW	<0.001	<0.005	<0.025	<0.05	NS	NS	NS
NM vs NW	NS	NS	NS	NS	NS	NS	NS
HM vs NM	NS	NS	<0.05	<0.005	NS	<0.005	NS
HW vs HM	<0.05	NS	NS	NS	NS	NS	NS

The number of vertical supported rears made during the first five minute interval of the 20 minute testing session for each age of testing from 22 to 50 days of age was as follows:

Day of Age	HM	NM	HW	NW
22	11.4	6.5	15.1	8.1
23	7.9	5.4	14.1	5.0
29	10.4	7.8	19.8	10.5
36	20.1	10.1	20.4	11.8
50	9.5	7.6	9.4	6.9

Those animals that had been handled during the nursing period demonstrated greater amounts of vertical supported activity than those that had not been handled. The greater vertical supported activity of the handled over the non-handled animals was statistically significant for each age except 50 days ($F = 15.56$; $df = 1, 60$; $p < 0.001$ for 22 days of age; $F = 16.44$; $df = 1, 60$; $p < 0.001$ for 23 days of age; $F = 16.63$; $df = 1, 60$; $p < 0.001$ for 29 days of age; $F = 26.62$; $df = 1, 60$; $p < 0.001$ for 36 days of age). The animals

that had nursed from dams fed high protein diets made significantly more supported vertical rears at 23 and 29 days of age ($F = 4.57$; $df = 1, 60$; $p < 0.05$ at 23 days of age and $F = 17.34$; $df = 1, 60$; $p < 0.001$ at 29 days of age). As was the case for the results of the other open field measures, the differences among the four groups for the initial five minutes were similar to those for the entire 20 minute testing period.

It is clear from Figure 4 that, in general, the age changes in the number of supported vertical rears conformed to the age changes already seen in horizontal activity, i.e., declining in all four groups from 22 to 23 days of age, increasing thereafter until 36 days of age, and declining again at 50 days of age. Although the relationships among the number of supported vertical rears for the four groups of animals was similar from 22 to 36 days of age, there was a significant interaction between handling, nutrition and age ($F = 5.36$; $df = 3, 54$; $p < 0.001$). This was probably the result of the much greater increase in supported rearing with age in the HM group than in either the HW or the NM groups.

Summary. Vertical rearing that was supported by the walls of the open field necessarily was carried out in the peripheral squares. It would therefore be expected that horizontal activity in the peripheral squares and vertical activity with support of the chamber walls would exhibit similar patterns. In general, this was found to be true, in that from 22 to 50 days of age, the two groups of handled animals demonstrated more of both of these activities than

did the non-handled animals. Whereas there were no consistent differences among the four groups in horizontal activity in the peripheral squares for those animals tested in adulthood (90 to 91 days of age), there were consistent differences among the four groups in the vertical supported activity at those ages, suggesting that the vertical activity measure is a more sensitive indicator of the long-term effects of early handling and nutritional status than is the horizontal activity measure.

Vertical activity not supported by chamber walls.

Table 5a and Figure 5 present the mean number of vertical rears that were not supported by the chamber walls made by each of the four groups at each age of testing. As was found for the other measures, the handled animals (HW and HM) made more unsupported vertical rears than the non-handled animals (NW and NM). This was statistically significant for each age of testing (Table 5b).

In all tests from 22 to 50 days of age, the HW animals made the greatest number of unsupported vertical rears and the NW animals the least number of unsupported vertical rears. This difference between the two well nourished groups was statistically significant for each day from 22 to 50 days (Table 5c). At 90 and 91 days of age, the HM group made the greatest number of unsupported vertical rears, and the NM animals the smallest number; this difference was statistically significant (Table 5c). At 22, 50, and 90 days of age, there was a significant interaction between nutrition and handling (Table 5b) which may be accounted for by the significant differences between

FIGURE 5

Number of Unsupported Vertical Rears

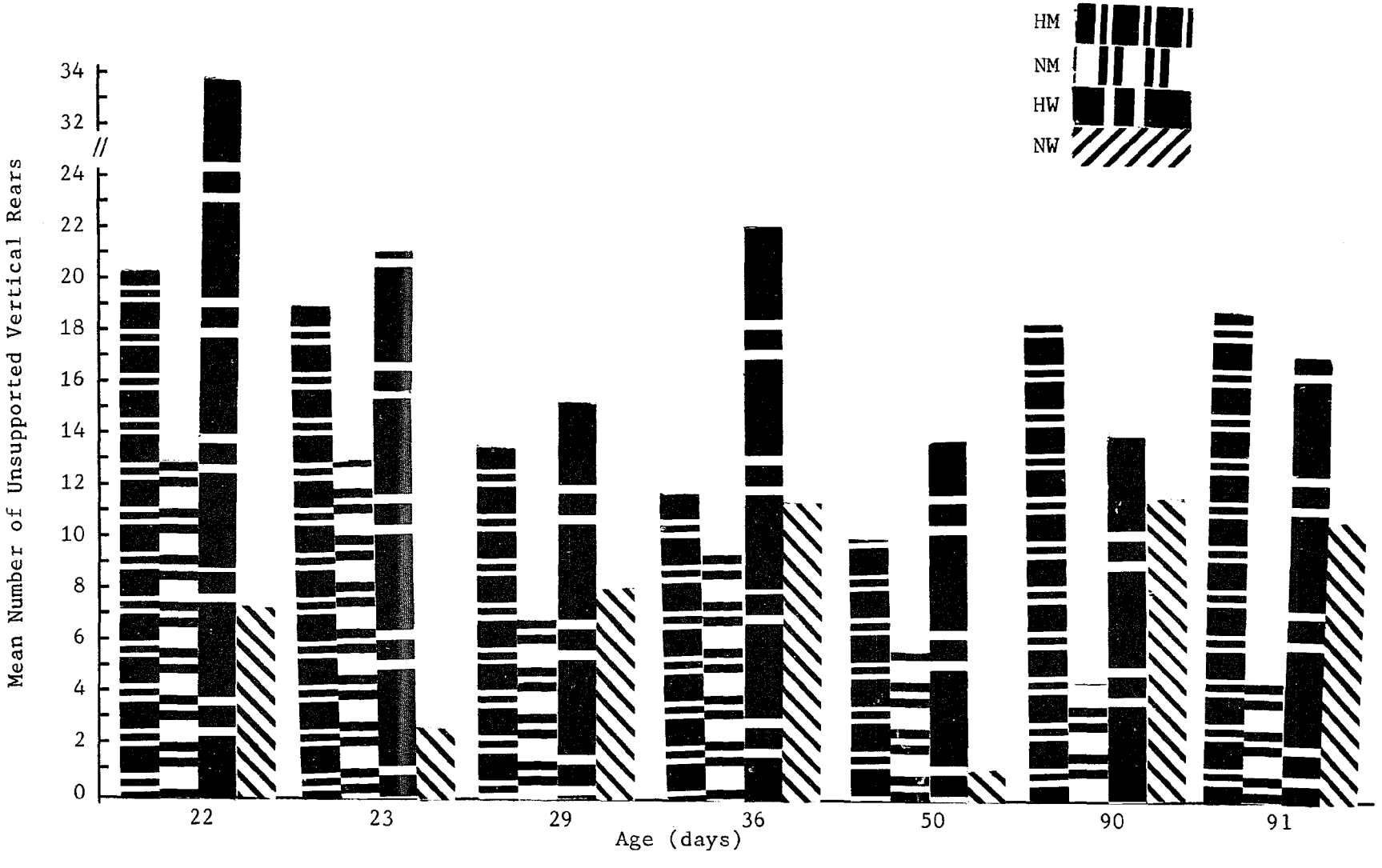

Table 5a

Number of Unsupported Vertical Rears

Age (days)		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
22	$\bar{X}$	20.3	12.9	33.9	7.1
	s.d.	13.6	10.9	15.4	10.8
23	$\bar{X}$	19.0	13.0	21.2	2.6
	s.d.	13.0	14.2	12.8	3.3
29	$\bar{X}$	13.5	6.8	15.5	7.8
	s.d.	7.1	6.6	14.1	8.8
36	$\bar{X}$	11.5	9.3	21.8	11.0
	s.d.	10.3	11.3	15.7	10.8
50	$\bar{X}$	9.8	5.1	13.6	0.87
	s.d.	6.0	4.4	8.1	1.8
90	$\bar{X}$	18.1	4.0	13.4	11.6
	s.d.	12.3	4.2	9.5	12.1
91	$\bar{X}$	18.9	3.7	16.3	10.1
	s.d.	14.1	3.7	13.7	9.0

Table 5b

ANOVAR for Unsupported Vertical Rears

Age (days)	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
22	Handling	1	4,658.06	4,658.06	27.68	<0.001
	Nutrition	1	248.06	248.06	1.47	NS
	Interact.	1	1,501.57	1,501.57	8.92	<0.005
	Error	60	10,096.25	168.27		
23	Handling	1	2,413.26	2,413.26	14.79	<0.001
	Nutrition	1	268.14	268.14	1.64	NS
	Interact.	1	631.27	631.27	3.87	NS
	Error	60	9,786.19	163.10		
29	Handling	1	777.60	777.60	8.38	<0.005
	Nutrition	1	35.26	35.26	0.37	NS
	Interact.	1	4.27	4.27	0.04	NS
	Error	60	5,567.40	92.79		
36	Handling	1	633.75	633.75	4.43	<0.05
	Nutrition	1	546.02	546.02	3.81	NS
	Interact.	1	277.35	277.35	1.93	NS
	Error	60	8,583.00	143.05		
50	Handling	1	612.50	612.50	19.93	<0.001
	Nutrition	1	0.50	0.50	0.01	NS
	Interact.	1	128.00	128.00	4.16	<0.05
	Error	28	860.50	30.73		
90	Handling	1	873.15	873.15	8.36	<0.01
	Nutrition	1	29.17	29.17	0.27	NS
	Interact.	1	511.01	511.01	4.89	<0.05
	Error	60	6,264.00	104.40		
91	Handling	1	1,397.20	1,397.20	10.68	<0.005
	Nutrition	1	43.39	43.39	0.33	NS
	Interact.	1	245.39	245.39	1.87	NS
	Error	60	7,848.60	130.81		

the number of unsupported vertical rears made by the two well-nourished groups at 22 and 50 days of age, and by the two malnourished groups at 90 days of age.

Table 5c

Post Hoc Comparisons for Unsupported Vertical Rears (p values)

	Age (days)						
	22	23	29	36	50	90	91
HW vs NW	<0.001	<0.005	NS	<0.05	<0.005	NS	NS
NM vs NW	NS	NS	NS	NS	NS	NS	NS
HM vs NM	NS	NS	NS	NS	NS	<0.01	<0.025
HW vs HM	<0.05	NS	NS	NS	NS	NS	NS

The number of unsupported vertical rears made by the four groups of animals during the first five minute time segment from 22 to 50 days of age were as follows:

Day of age	HM	NM	HW	NW
22	4.2	2.2	9.1	1.6
23	4.1	2.8	4.4	1.0
29	2.0	0.8	4.1	1.1
36	1.7	2.7	4.2	1.7
50	4.0	0.9	3.8	0.3

The animals that had been handled in early life made significantly more unsupported vertical rears than the non-handled animals during the first five minute time segment at all ages except 36 days of age ($F = 16.59$; $df = 1, 60$; $p < 0.001$ at 22 days of age; $F = 6.56$; $df = 1, 60$; $p < 0.025$ at 23 days of age; $F = 5.55$; $df = 1, 60$;

$p < 0.025$ at 29 days of age; $F = 15.53$; $df = 1, 28$; $p < 0.001$ at 50 days of age). Whereas the HW animals consistently made more unsupported rears than the other three groups, none of the differences between the well nourished and the malnourished animals were statistically significant. As with the other open-field measures, the differences among the four groups over the entire 20 minutes of testing were evident during the first five minutes.

Figure 5 shows the age changes in unsupported vertical rearing. As had been the case for the other measures, all groups except the NM group decreased their unsupported vertical rearing from 22 to 23 days of age. However, in contrast to the findings for the other open field measures, there was a further decrease in the number of unsupported vertical rears made from 23 to 29 days of age in all groups except the NW group, and a subsequent increase in this behavior from 29 to 36 days of age in all groups except the HM group. Finally, similar to the findings for the other measures, in the two week interval between 36 and 50 days of age, the number of unsupported vertical rears decreased in all four groups, with that in the two well nourished groups (HW and NW) declining more than that in the two malnourished groups (HM and NM). As had been the case for the other measures, there was a significant nutrition, handling, and age interaction ($F = 3.84$; $df = 3, 54$; $p < 0.01$). This may have resulted from the fact that the NW animals had more unsupported rears at 36 days of age than they had at 22 while the other three groups of

animals had fewer; or it may have resulted from the more pronounced change with age in the HW group than in the HM group.

Summary. Unsupported vertical rearing occurred mostly in the central squares and the differences in unsupported vertical rearing among the four groups was similar to the group differences found for the number of central squares entered. The early-handled animals were significantly more active than the non-handled animals, with the HW animals being the most active at each age of testing.

Entries into the "Novel" Square.

Table 6a and Figure 6 present the mean number of entries into the square in which a ball was suspended for each of the four groups. From 22 to 50 days of age, the same relative pattern of difference among the four groups was evident as had been evident for the other open field measures. At each age, except 90 days of age, the HW animals entered this square on more occasions than any of the other three groups. The HM animals followed next in total number of entries at all ages with the two groups of non-handled animals (NW and NM) entering the novel square significantly fewer times than the handled animals (HW and HM) at all ages except at 91 days (Table 6b). Since the HW animals entered the novel square significantly more times than the NW animals at 22, 23, 29, 36, and 50 days of age (Table 6c), when the two groups of well-nourished animals were considered together, they only entered the novel square significantly more times than the malnourished animals at 22 days of age, and

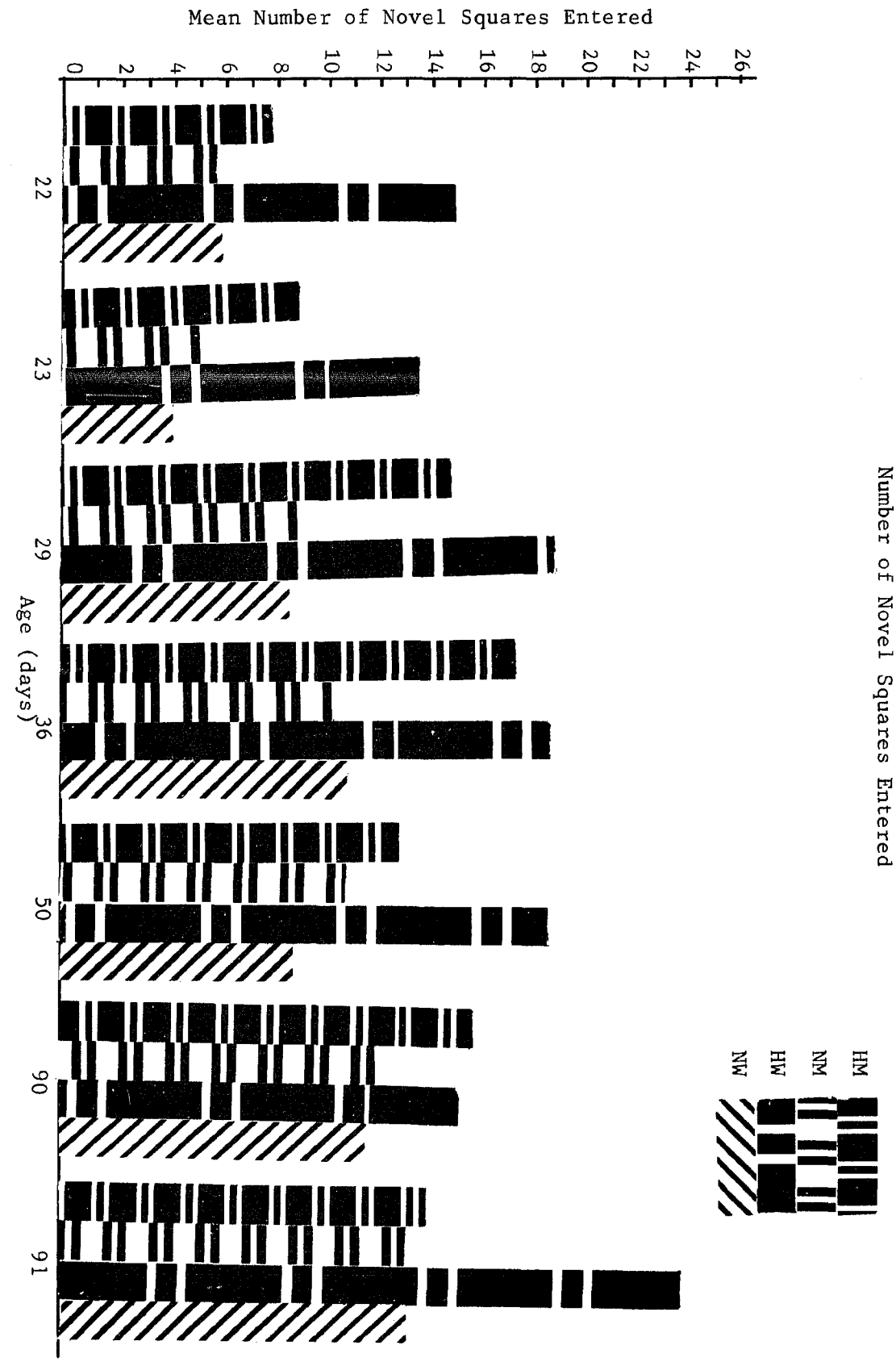

FIGURE 6

Number of Novel Squares Entered

Table 6a

Number of Novel Squares Entered

Age (days)		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
22	$\bar{X}$	7.9	5.7	15.0	5.8
	s.d.	4.2	2.7	3.8	3.3
23	$\bar{X}$	8.8	4.8	13.5	3.8
	s.d.	9.5	3.4	4.0	3.0
29	$\bar{X}$	14.9	8.5	18.8	8.2
	s.d.	6.3	6.0	6.5	6.4
36	$\bar{X}$	17.0	10.0	18.3	10.5
	s.d.	6.2	7.8	4.5	8.9
50	$\bar{X}$	12.8	10.5	18.3	8.0
	s.d.	5.3	5.6	3.5	6.9
90	$\bar{X}$	15.7	11.9	14.9	11.3
	s.d.	4.5	6.0	6.7	7.8
91	$\bar{X}$	13.8	13.0	24.0	13.6
	s.d.	7.4	4.6	27.0	5.3

Table 6b

ANOVAR for Novel Squares Entered

Age (days)	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
22	Handling	1	529.00	529.00	41.81	<0.001
	Nutrition	1	203.06	203.06	16.05	<0.001
	Interact.	1	196.00	196.00	15.49	<0.001
	Error	60	759.38	12.65		
23	Handling	1	763.14	763.14	22.80	<0.001
	Nutrition	1	54.39	54.39	1.62	NS
	Interact.	1	129.39	129.39	3.86	NS
	Error	60	2,008.44	33.47		
29	Handling	1	1,092.27	1,092.27	27.38	<0.001
	Nutrition	1	52.27	52.27	1.31	NS
	Interact.	1	64.06	64.06	1.60	NS
	Error	60	2,393.40	39.89		
36	Handling	1	821.40	821.40	16.56	<0.001
	Nutrition	1	11.27	11.27	0.22	NS
	Interact.	1	2.40	2.40	0.04	NS
	Error	60	2,974.80	49.58		
50	Handling	1	312.50	312.50	10.55	<0.005
	Nutrition	1	18.00	18.00	0.60	NS
	Interact.	1	128.00	128.00	40.79	<0.001
	Error	28	829.00	29.61		
90	Handling	1	193.79	193.79	4.57	<0.05
	Nutrition	1	6.47	6.47	0.15	NS
	Interact.	1	0.046	0.046	0.00	NS
	Error	60	2,542.20	42.37		
91	Handling	1	378.82	378.82	1.44	NS
	Nutrition	1	356.54	356.54	1.36	NS
	Interact.	1	283.88	283.88	1.08	NS
	Error	60	15,724.20	262.07		

there was a statistically significant interaction between handling and nutrition at 22 and 50 days of age (Table 6b).

In order to assess the contribution of novel square entries during the first five minutes to the differences among the four groups of animals for the 20 minute testing session, the number of entries into the novel square were analysed for the first five minute interval. The data for 22 to 50 days of age were as follows;

Table 6c

Post Hoc Comparisons for Novel Squares Entered (p values)

	Age (days)						
	22	23	29	36	50	90	91
HW vs NW	<0.001	<0.001	<0.001	<0.05	<0.005	NS	NS
NM vs NW	NS	NS	NS	NS	NS	NS	NS
HM vs NM	NS	NS	<0.05	NS	NS	NS	NS
HW vs HM	<0.001	NS	NS	NS	NS	NS	NS

Day of age	HM	NM	HW	NW
22	3.9	1.9	5.6	2.3
23	2.7	1.3	5.3	1.9
29	4.6	3.2	8.0	3.6
36	7.2	4.7	8.0	3.8
50	5.0	4.8	6.3	3.0

During the first five minute interval at all ages except 50 days, those animals that had been handled during the nursing period entered the novel square a significantly greater number of times than those that had not been handled: (F = 20.06; df = 1, 60;

$p < 0.001$ at 22 days of age; $F = 26.86$; $df = 1, 60$; $p < 0.001$ at 23 days of age; $F = 17.86$; $df = 1, 60$ $p < 0.001$ at 29 days of age; $F = 19.64$; $df = 1, 60$; $p < 0.001$ at 36 days of age). The well nourished animals entered the novel square significantly more times than the malnourished animals during the first five minute interval only at 23 ($F = 11.62$; $df = 1, 60$; $p < 0.005$) and 29 days of age ($F = 7.27$; $df = 1, 60$; $p < 0.01$). Therefore, the differences among groups in the number of entries into the novel square for the 20 minute testing session was already evident during the first five minutes.

Figure 6 illustrates the age changes that took place in the number of novel square entries. As already seen in most of the other open field measures monitored, there was a decrease in the number of novel square entries from 22 to 23 days of age in all but the HM group, with successive increases thereafter until 36 days of age (except for the HW group which decreased from 29 to 36 days of age). The two malnourished groups (HM and NM) made fewer entries into the novel square at 50 than at 36 days of age.

Summary. The differences among the groups in number of entries into the novel square followed the same trends observed for all other behavioral measures. The handled animals made more entries than the non-handled animals at all ages tested, and the handled well-nourished animals made more entries than the malnourished animals from 22 to 50 days of age. The number of novel square entries increased from 22 to 50 days in all four groups.

Duration of attention to "Novel Object".

Analysis of the amount of time spent actively attending to (pawing, chewing, etc.) the ball ("novel object") in the corner square accentuated some of the differences among the four groups. As may be seen in Table 7a and Figure 7, the two groups of handled animals (HW and HM) spent more time attending to the ball than did the two groups of non-handled animals (NW and NM) at all ages tested from 22 to 50 days of age. This difference between the handled and the non-handled animals was statistically significant at each of these ages (Table 7b). The HW animals spent significantly more time pawing and chewing the rubber ball than the NW animals (Table 7c) on all tests from 22 to 50 days of age. Since the HW animals spent

Table 7c

Post Hoc Comparisons for Duration of Attention to Novel Object
(p values)

	Age (days)						
	22	23	29	36	50	90	91
HW vs NW	<0.001	<0.001	<0.001	<0.001	<0.005	NS	NS
NM vs NW	NS	NS	NS	NS	NS	NS	NS
HM vs NM	NS	NS	NS	NS	NS	NS	NS
HW vs HM	<0.001	<0.001	NS	NS	<0.025	NS	NS

significantly more time engaged in this behavior than the HM animals at 22, 23, and 50 days of age (Table 7c) the difference between the well nourished and the malnourished animals was significant only at these ages.

FIGURE 7

Duration of Attention to Novel Object (seconds)

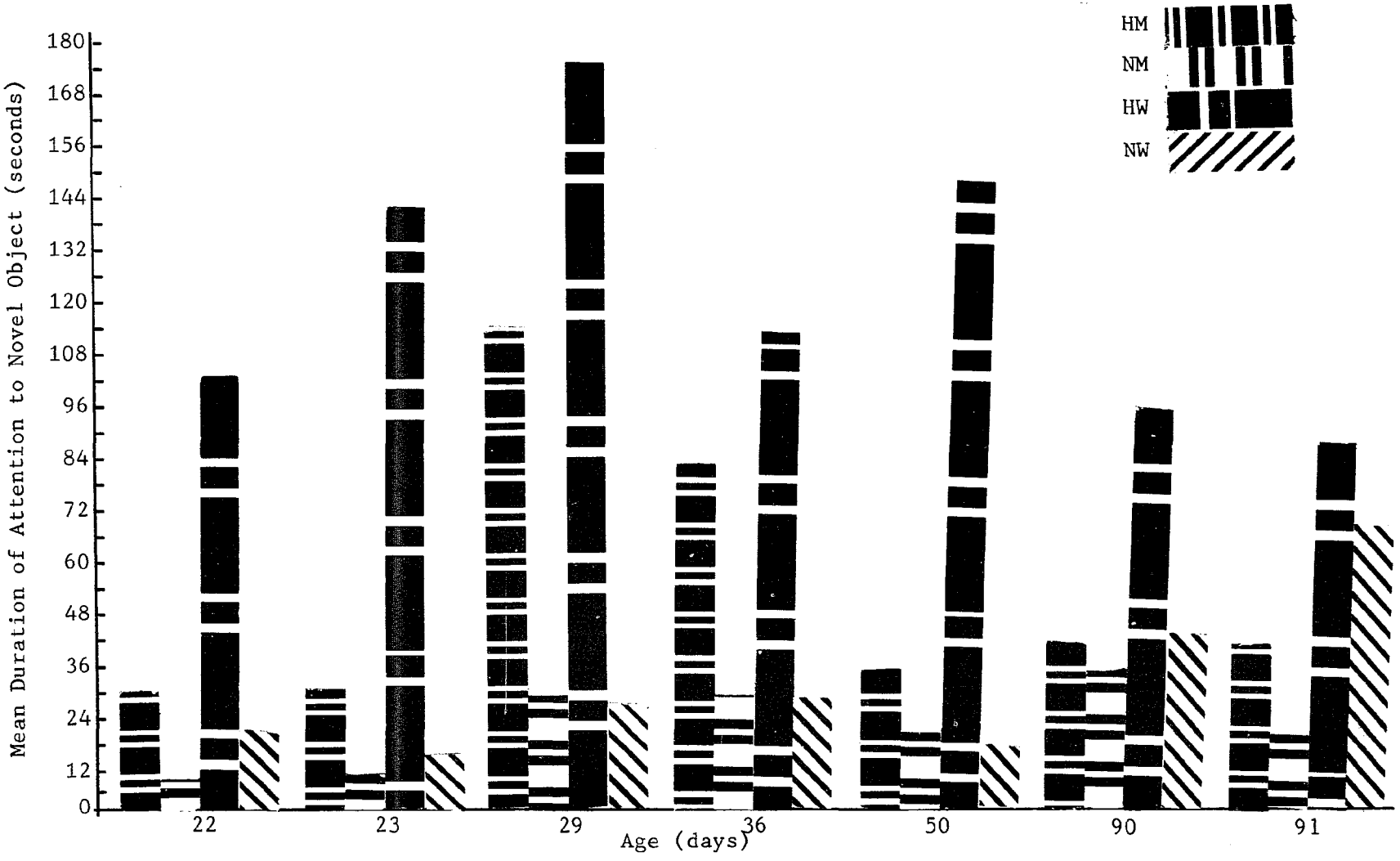

Table 7a

Duration of Attention to Novel Object (seconds)

Age (days)		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
22	$\bar{X}$	30.1	8.8	103.4	21.1
	s.d.	35.2	7.0	58.0	39.8
23	$\bar{X}$	30.6	11.6	141.9	15.3
	s.d.	31.6	12.7	100.5	27.3
29	$\bar{X}$	115.0	29.9	174.9	28.1
	s.d.	77.2	34.1	105.8	24.2
36	$\bar{X}$	82.7	31.0	112.7	31.1
	s.d.	53.6	28.2	57.4	40.1
50	$\bar{X}$	34.4	21.2	148.1	15.1
	s.d.	9.5	17.4	128.2	17.6
90	$\bar{X}$	44.8	35.0	97.1	42.3
	s.d	26.9	33.6	117.9	59.3
91	$\bar{X}$	42.8	23.5	91.8	73.3
	s.d.	47.2	18.9	105.8	92.0

Table 7b

ANOVAR for Duration of Attention to Novel Object

Age (days)	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
22	Handling	1	42,952.56	42,952.56	26.66	$<$0.001
	Nutrition	1	29,412.25	29,412.25	18.25	$<$0.001
	Interact.	1	14,884.00	14,884.00	9.23	$<$0.005
	Error	60	96,663.63	1,611.06		
23	Handling	1	84,826.56	84,826.56	27.83	$<$0.001
	Nutrition	1	52,900.00	52,900.00	17.35	$<$0.001
	Interact.	1	46,332.56	46,332.56	15.20	$<$0.001
	Error	60	182,855.88	3,047.59		
29	Handling	1	201,608.07	201,608.07	22.35	$<$0.005
	Nutrition	1	12,673.07	12,673.07	1.40	NS
	Interact.	1	14,229.60	14,229.60	1.57	NS
	Error	60	541,025.40	9,017.09		
36	Handling	1	66,533.40	66,533.40	29.76	$<$0.001
	Nutrition	1	3,405.07	3,405.07	1.52	NS
	Interact.	1	3,345.07	3,345.07	1.49	NS
	Error	60	134,113.20	2,235.22		
50	Handling	1	42,705.00	42,705.00	9.96	$<$0.005
	Nutrition	1	23,166.00	23,166.00	5.40	$<$0.05
	Interact	1	28,740.00	28,740.00	6.70	$<$0.025
	Error	28	120,030.00	4,286.80		
90	Handling	1	14,938.00	14,938.00	2.84	NS
	Nutrition	1	12,699.00	12,699.00	2.41	NS
	Interact.	1	7,231.60	7,231.60	1.37	NS
	Error	60	315,414.00	5,256.90		
91	Handling	1	4,324.50	4,324.50	0.68	NS
	Nutrition	1	29,713.00	29,713.00	4.73	$<$0.05
	Interact.	1	1.81	1.81	0.00	NS
	Error	60	376,650.00	6,277.50		

The pattern of differences in the amount of time spent attending to the novel object among the groups tested for the first time at 90 days of age was somewhat different than that found for the animals that were tested from 22 to 50 days of age. Although the HW animals tested from 22 to 50 days of age and those tested at 90 and 91 days of age spent the most time attending to the novel object, the relationship among the other three groups of animals was different for those tested at 90 and 91 days of age than it had been for those tested during infancy (Table 7a). Although the well nourished animals (HW and NW) spent more time attending to the novel object at 91 days of age than did the malnourished animals (HM and NM) there were no significant differences between the handled and non-handled animals at 90 or 91 days of age as there had been in the earlier testing (Table 7b). Moreover, none of the differences between any two groups tested at 90 and 91 days were statistically significant (Table 7c).

The average duration of attention to the novel object per entry into the novel square for the four groups of animals tested in adulthood was somewhat different than that for those animals tested during infancy (Table 8a). Whereas the handled animals tested immediately after weaning demonstrated significantly more attention upon each entry regardless of their early nutritional status, the well-nourished animals that were tested in later life engaged the ball for longer times upon each entry regardless of whether they had been handled (Table 8b).

The amount of time (in seconds) spent attending to the novel object during the first five minute time segment from 22 to 50 days of age was as follows:

Day of age	HM	NM	HW	NW
22	9.0	3.5	19.8	4.8
23	8.4	3.9	31.0	3.1
29	18.6	9.7	25.7	7.3
36	25.9	7.4	14.3	11.7
50	23.1	3.8	15.9	3.3

The greater amount of time spent attending to the novel object by the handled than the non-handled animals was statistically significant for this interval of testing from 22 to 50 days of age ($F = 14.80$; $df = 1, 60$; $p < 0.001$ at 22 days of age; $F = 22.36$; $df = 1, 60$; $p < 0.001$ at 23 days of age; $F = 10.56$; $df = 1, 60$; $p < 0.005$ at 29 days of age; $F = 11.88$; $df = 1, 60$; $p < 0.005$ at 36 days of age; $F = 10.26$; $df = 1, 28$; $p < 0.005$ at 50 days of age). The greater amount of time spent attending to the novel object by the well-nourished than the malnourished animals was statistically significant during the first five minutes at 22, 23, and 50 days of age ($F = 5.17$; $df = 1, 60$; $p < 0.025$ at 22 days of age; $F = 10.00$; $df = 1, 60$; $p < 0.005$ at 23 days of age; $F = 8.34$; $df = 1, 28$; $p < 0.01$ at 50 days of age). As with the other measures, the group differences for the first five minutes of testing were the same as those for the entire 20 minute testing session.

The age changes in the amount of time spent attending to the novel object were different from the age changes in the other

measures: Only one group (NW) spent less time attending to the novel object at 23 days of age than they had at 22 days of age (Figure 7). As had been the case for the other measures, although all four groups spent more time engaged in this activity at 29 than at 23 days of age, the two handled groups (HW and HM) spent less time at 36 days whereas the two non-handled groups (NW and NM) did not change. Finally, only the HW group pawed and chewed at the rubber ball for a longer time at 50 days of age than they had two weeks earlier, while the other three groups did so for shorter times. While the HW animals spent more time pawing and chewing the rubber ball at all ages tested, they demonstrated the smallest increase in this activity from 22 to 36 days of age. This was in contrast to the greater increase in attention to the novel object by the HM animals with increase in age, resulting in a significant interaction between age, nutrition and handling ($\underline{F} = 2.90$; $\underline{df} = 3, 54$; $\underline{p} < 0.05$).

Table 8a presents the mean amount of time spent attending to the novel object for the 20 minute testing period divided by the number of entries into the novel square for each animal, and gives an indication of the amount of time spent attending to the rubber ball each time the square was entered.[1] On each day of testing from 22 to 50

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1. While the two groups of handled animals (especially the HW group) would often repeatedly engage the rubber ball in their teeth, walk to one of the three other corners of the open field, release the ball, and visually follow its bounce against the chamber walls, the two groups of non-handled animals often reversed their direction of locomotion when approaching the ball, or accelerated their pace while traversing through the square with the ball in it. They would also occasionally remain immobile behind the ball.

days of age, not only were the handled animals more likely to enter the novel square (Table 6a), but once they entered the square, they were more likely to remain there and manipulate the rubber ball (Table 8a). The handled animals spent significantly more time pawing and chewing the ball during each individual entry into the square than the non-handled animals from 22 to 50 days of age (Table 8b). Further, the HW animals spent significantly more time attending to the ball than the NW animals upon each entry into the square at 22, 23, 29, and 50 days of age and significantly more time than the HM animals at 22 and 23 days of age (Table 8c). The greater likelihood of the HW animals remaining in the novel square and manipulating the

Table 8c

Post Hoc Comparison for Duration of Attention per Novel Square Entry (p values)

	Age (days)						
	22	23	29	36	50	90	91
HW vs NW	<0.005	<0.001	<0.05	NS	<0.005	NS	NS
NM vs NW	NS	NS	NS	NS	NS	NS	NS
HM vs NM	NS	NS	NS	NS	NS	NS	NS
HW vs HM	<0.005	<0.005	NS	NS	NS	NS	NS

ball each time they entered it contributed to the significantly greater average duration of attention per entry of the well-nourished animals as well as to the statistically significant interaction between handling and nutrition at 22 and 23 days of age (Table 8b).

Table 8a

Duration of Attention per "Novel Square" Entry

Age (days)		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
22	$\bar{X}$	2.8	1.5	7.0	2.6
	s.d.	2.7	1.0	3.8	3.5
23	$\bar{X}$	3.3	2.3	10.0	2.6
	s.d.	3.5	2.8	6.8	4.0
29	$\bar{X}$	6.7	2.5	11.6	2.3
	s.d.	4.1	2.2	13.6	2.1
36	$\bar{X}$	4.6	3.0	6.0	3.6
	s.d.	2.6	3.1	2.9	4.3
50	$\bar{X}$	3.3	1.5	8.0	1.3
	s.d.	2.2	1.2	6.0	1.1
90	$\bar{X}$	2.9	2.8	5.9	4.0
	s.d.	1.8	1.9	4.4	3.4
91	$\bar{X}$	2.4	1.9	4.4	6.3
	s.d.	2.1	0.95	4.6	7.8

Table 8b

ANOVAR for Duration of Attention per "Novel Square" Entry

Age (days)	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
22	Handling	1	129.19	129.19	14.49	<0.001
	Nutrition	1	110.80	110.80	12.43	<0.001
	Interact.	1	39.17	39.17	4.40	<0.05
	Error	60	534.37	8.91		
23	Handling	1	304.90	304.90	14.84	<0.001
	Nutrition	1	218.86	218.86	10.65	<0.005
	Interact.	1	189.99	189.99	9.24	<0.005
	Error	60	1,232.20	20.54		
29	Handling	1	686.38	686.38	13.17	<0.001
	Nutrition	1	80.30	80.30	1.54	NS
	Interact.	1	97.57	97.57	1.87	NS
	Error	60	3,126.00	52.10		
36	Handling	1	62.40	62.40	5.69	<0.025
	Nutrition	1	14.57	14.57	1.32	NS
	Interact.	1	2.82	2.82	0.25	NS
	Error	60	659.60	10.96		
50	Handling	1	143.35	143.35	12.83	<0.001
	Nutrition	1	39.38	39.38	3.52	NS
	Interact.	1	46.53	46.53	4.16	<0.05
	Error	28	312.76	11.17		
90	Handling	1	16.40	16.40	1.60	NS
	Nutrition	1	63.30	63.30	6.19	<0.025
	Interact.	1	11.69	11.69	1.14	NS
	Error	60	612.60	10.21		
91	Handling	1	4.95	4.95	0.21	NS
	Nutrition	1	122.92	122.92	5.45	<0.025
	Interact.	1	17.78	17.78	0.78	NS
	Error	60	1,351.20	22.52		

Summary. There were greater differences among the four groups of animals in the duration of attention to the novel object than in the other open field measures. The handled animals spent more time pawing and chewing the ball on each day of testing from 22 to 50 days of age; and the well-nourished animals spent more time than the malnourished animals at 22, 23, 50, and 91 days of age. As was the case for the other open field measures, group differences in attention to the novel object during the 20 minute testing session were observed during the first five minutes at each age.

Summary of results of behavioral measures in the open field.

Although six different behaviors were measured in the open field during the course of early development, and again in adulthood, the nature and direction of differences among the four groups for all measures were basically the same with only minor variations. The major finding was that the handled animals were more active than the non-handled animals, and this difference in activity was more pronounced for those animals nursed by dams fed a high protein diet. Another persistent finding was that the well-nourished animals which were not handled were usually less active than any of the other groups. This differential effect of handling on the malnourished and well-nourished animals often resulted in an interaction between handling and nutrition. In general, the amounts of all open field activities for each group of animals increased with increase in age, reaching an asymptote or decreasing by 50 days of age. Furthermore, the differences in activity among the four groups of animals for

the 20 minute testing session were generally established during the first five minutes. The one set of results which was not as consistent across the various open-field measures was obtained from the animals tested at 90 and 91 days of age. The pattern of differences among the groups tested in adulthood was often different from that found for the animals tested in earlier life. In addition, there were some differences between the findings at 90 days of age and at 91 days of age. Despite these inconsistencies, the long-term effect of handling was evidenced by the fairly consistent finding of greater amounts of activity in the handled animals compared to the non-handled animals.

Qualitative analysis of open field behavior.

In addition to the finding of an effect of both early handling and early nutritional status on the quantitative measures of activity in the open field, the qualitative pattern of activity was also affected. The two groups of handled animals, especially the HW group, maintained a relatively steady pace in moving about the open field throughout the testing periods. In contrast, the NM animals alternated short periods of rapid locomotion with periods of inactivity, and the NW animals usually alternated periods of slow steady locomotion with periods of inactivity. For both non-handled groups, activity occurred primarily on the peripheral squares. These observations are supported by the finding of low levels of activity in the NW animals as well as by the greater ratio of peripheral to

central square entries of these animals than the other three groups.

Somatometric Measures

Analysis of the body weights of both dams and pups on the day of parturition failed to reveal any statistically significant differences among the four groups. The mean weights (in grams) of the dams were as follows: HM = 240.0; NM - 236.2; HW = 241.9; NW = 243.1. The mean body weights of the pups in the various groups were: HM = 6.61; NM - 6.69; HW = 6.67; NW = 6.58.

At weaning, the body weights of those dams whose pups had been handled during the nursing period were slightly, but not significantly, greater than the body weights of those dams whose pups had not been handled (Tables 9a and 9b). However, the body weights of those dams fed high protein diets were significantly greater than those of dams fed low protein diets not only when the two high protein groups were compared to the two low protein groups (Table 9b), but also when each high protein group was compared to its respective low protein group (Table 9c).

At weaning, the direction of differences in the body weights of the pups in the four experimental groups paralleled those of their dams. Those pups that had been handled weighed significantly more than those that had not been handled (Tables 9a and 9b), but this difference was probably due, at least in part, to the fact that the HW group was significantly heavier than each of the other three groups (Table 9c). The weights of the pups whose

Table 9a

Somatometric Measures

Measure		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
Dams' Body Weights at Weaning (g)	$\bar{X}$	214.37	195.19	305.02	268.57
	s.d.	32.23	33.91	19.10	17.61
Subjects' Body Weights at Weaning (g)	$\bar{X}$	22.81	20.80	57.55	49.10
	s.d.	5.85	4.25	10.51	7.72
Subjects' Body Weights at 55 days	$\bar{X}$	171.87	157.17	218.54	224.23
	s.d.	35.78	26.85	48.40	39.74
Subjects' Forebrain Weights at 55 days	$\bar{X}$	1.15	1.12	1.26	1.21
	s.d.	0.08	0.09	0.12	0.06
$\frac{\text{Forebrain Weight}}{\text{Body Weight} \times 10^4}$	$\bar{X}$	69.17	73.68	66.19	56.13
	s.d.	11.61	16.54	17.11	8.53
Brainstem Weight at 55 days	$\bar{X}$	0.19	0.19	0.22	0.22
	s.d.	0.02	0.18	0.03	0.03
$\frac{\text{Brainstem Weight}}{\text{Body Weight} \times 10^4}$	$\bar{X}$	11.58	13.20	10.88	9.39
	s.d.	2.38	2.80	2.01	1.92
Cerebellum Weight at 55 days	$\bar{X}$	0.21	0.21	0.25	0.26
	s.d.	0.03	0.02	0.02	0.02

Table 9a (cont.)

Somatometric Measures

Measure		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
Cerebellum Weight / Body Weight x 10^4	$\bar{X}$	12.69	14.06	12.80	11.61
	s.d.	1.88	2.98	2.01	1.54
Liver Weight at 55 days	$\bar{X}$	8.54	7.65	9.41	10.12
	s.d.	2.22	1.90	2.79	2.40
Liver Weight / Body Weight	$\bar{X}$	49.37	48.02	47.27	43.84
	s.d.	4.07	6.07	12.78	8.43
Adrenal Weight at 55 days	$\bar{X}$	3.76	3.23	5.18	4.08
	s.d.	0.64	0.73	1.07	1.08
Adrenal Weight / Body Weight x 10^4	$\bar{X}$	228.87	243.39	266.19	193.58
	s.d.	63.38	85.22	63.83	66.49

Table 9b

ANOVAR for Somatometric Measures

	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
Dams' Body Weights at Weaning	Handling	1	3,099.70	3,099.70	4.32	NS
	Nutrition	1	26,921.00	26,921.00	37.60	< 0.001
	Interact.	1	296.70	296.70	0.41	NS
	Error	12	8,591.00	715.92		
Subjects' Body Weights at Weaning	Handling	1	845.23	845.23	15.00	< 0.001
	Nutrition	1	30,695.00	30,695.00	545.01	< 0.001
	Interact.	1	320.90	320.90	5.69	< 0.025
	Error	120	6,757.90	56.32		
Subjects' Body Weights at 55 days	Handling	1	278.49	278.49	0.17	NS
	Nutrition	1	44,356.00	44,356.00	28.33	< 0.001
	Interact.	1	1,425.10	1,425.10	0.91	NS
	Error	60	93,282.00	1,554.70		
Forebrain Weights at 55 days	Handling	1	0.0351	0.0351	4.57	< 0.05
	Nutrition	1	0.1664	0.1664	21.65	< 0.001
	Interact.	1	0.000239	0.000239	0.03	NS
	Error	60	0.4614	0.00769		
$\frac{\text{Forebrain Weight}}{\text{Body Weight} \times 10^4}$	Handling	1	103.94	103.94	0.53	NS
	Nutrition	1	1,425.10	1,425.10	7.33	< 0.01
	Interact.	1	718.08	718.08	3.69	NS
	Error	60	11,650.80	194.18		

Table 9b (cont.)

ANOVAR for Somatometric Measures

	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
Brainstem Weight at 55 days	Handling	1	0.0361	0.0361	0.64	NS
	Nutrition	1	0.0807	0.0807	1.44	NS
	Interact.	1	0.0344	0.0344	0.06	NS
	Error	60	3.334	0.0556		
Brainstem Weight / Body Weight x 10^4	Handling	1	0.0567	0.0567	0.00	NS
	Nutrition	1	68.79	68.79	13.46	< 0.001
	Interact.	1	32.46	32.46	6.35	< 0.025
	Error	60	306.582	5.11		
Cerebellum Weight at 55 days	Handling	1	0.0000181	0.0000181	0.38	NS
	Nutrition	1	0.0228	0.0228	48.40	< 0.001
	Interact.	1	0.000000694	0.000000694	0.00	NS
	Error	60	0.028	0.00047		
Cerebellum Weight / Body Weight x 10^4	Handling	1	0.111	0.111	0.02	NS
	Nutrition	1	18.27	18.27	4.07	< 0.05
	Interact	1	22.11	22.11	4.93	< 0.05
	Error	60	268.80	4.48		
Liver Weight at 55 days	Handling	1	0.116	0.116	0.02	NS
	Nutrition	1	38.13	38.13	6.66	< 0.025
	Interact.	1	8.79	8.79	1.54	NS
	Error	60	343.40	5.72		

Table 9b (cont.)

ANOVAR for Somatometric Measures

	Source of Variation	d.f.	Sums of Squares	Mean Square	F	p
Liver Weight / Body Weight	Handling	1	80.10	80.10	1.01	NS
	Nutrition	1	138.00	138.00	1.57	NS
	Interact.	1	15.13	15.13	0.19	NS
	Error	60	4,714.38	78.57		
Adrenal Weight at 55 days	Handling	1	0.000945	0.000945	11.09	< 0.005
	Nutrition	1	0.00185	0.00185	21.75	< 0.001
	Interact.	1	0.000111	0.000111	1.31	NS
	Error	60	0.00511	0.000085		
Adrenal Weight / Body Weight x 10^4	Handling	1	12,058.00	12,058.00	2.44	NS
	Nutrition	1	557.69	557.69	0.11	NS
	Interact.	1	27,138.00	27,138.00	5.51	< 0.025
	Error	60	295,464.40	4,924.40		

Table 9c

Post Hoc Comparisons for Somatometric Measures
(p values)

	Group			
	HW vs NW	NM vs NW	HM vs NM	HW vs HM
Dams' Body Weights at Weaning	NS	< 0.025	NS	< 0.005
Subjects' Body Weights at Weaning	< 0.001	< 0.001	NS	< 0.001
Subjects' Body Weights at 55 days	NS	< 0.001	NS	< 0.025
Forebrain Weight at 55 days	NS	< 0.05	NS	< 0.025
Forebrain Weight/Body Weight x 10^4	NS	< 0.01	NS	NS
Brainstem Weights at 55 days	NS	NS	NS	NS
Brainstem Weight/Body Weight x 10^4	NS	< 0.001	NS	NS
Cerebellum Weights at 55 days	NS	< 0.001	NS	< 0.001
Cerebellum Weight/Body Weight x 10^4	NS	< 0.05	NS	NS
Liver Weight at 55 days	NS	NS	NS	NS
Liver Weight/Body Weight	NS	NS	NS	NS
Adrenal Weight at 55 days	< 0.05	NS	NS	< 0.005
Adrenal Weight/Body Weight x 10^4	NS	NS	NS	NS

dams were fed the low protein diet were less than one half the weights of the pups whose dams were fed the high protein diet (Table 9a). As expected, the weight difference between the well-nourished and the malnourished pups was highly significant (Table 9b), as was the greater weight of each of the well nourished groups when compared to its respective malnourished group (Table 9c).

With only one reversal, the direction of difference in body weight among the four groups of animals was the same when they were killed for chemical analysis at 55 days of age as it had been at weaning. Those animals that had nursed from dams fed high protein diets were still heavier than those that had nursed from dams fed low protein diets (Table 9a) and this difference was statistically significant (Table 9b). In addition, the weight difference between the HW and the HM animals and that between the NW and the NM animals (Table 9c) was also maintained. Although the HM animals were heavier than the NM animals at 55 days of age, as they had been at weaning, the HW animals were no longer heavier than the NW animals (Table 9a).

In general, the group differences in the absolute weights of the forebrains, brainstems, cerebelli, livers, and adrenal glands of those animals killed for chemical analysis at 55 days of age were similar to the group differences in body weight. Except for the brainstem, the organs of the animals that had nursed from the well-nourished dams were significantly heavier than those of animals that had nursed from the malnourished dams (Tables 9a and 9b).

The forebrains and cerebelli of the HW animals were significantly heavier than those of the NM animals, and the forebrains, cerebelli and adrenal glands of the HW animals were significantly heavier than those of the HM animals (Table 9c). The group differences in forebrain and adrenal gland weight also parallel those for body weight. Those animals that had been handled early in life had significantly heavier forebrains and adrenal glands than those animals that had not been handled (Table 9b), and the adrenal glands of the HW animals were significantly heavier than those of the NW animals (Table 9c).

In order to assess the effects of early nutritional status and early handling on the organ weight: body weight ratios, the absolute weight of each organ was divided by the animal's body weight. This measure yields different information than the absolute organ weight since absolute weights could vary merely as a function of body weight. With the exception of the adrenal gland and cerebellum for the handled animals, all organs of the animals that had nursed from malnourished dams were heavier with respect to body weights than those of the animals that had nursed from well-nourished dams. The forebrain, brainstem and cerebellar weights of the malnourished animals were significantly greater with respect to body weight than were those of the well-nourished animals (Table 9b). The forebrain, brainstem, and cerebellar weights of the NM animals with respect to their body weights were significantly greater in comparison to these organ-to-body weight ratios of the NW animals.

The forebrain, brainstem, cerebellum, liver, and adrenal gland weights with respect to body weight were greater in the HW group than in the NW group (Table 9a). In contrast, the forebrain, brainstem, cerebellar and adrenal gland weights with respect to body weight were greater in the NM group than in the HM group (Table 9a). In no case was there a statistically significant difference between the handled animals (HW and HW) and the non-handled animals (NW and NM), and there was a statistically significant interaction between handling and nutrition for the brainstem, cerebellum, and adrenal gland weight: body weight ratios (Table 9b). No significant differences were found when each handled group was compared to its respective non-handled group (Table 9c).

Summary. Protein restriction to lactating dams resulted in a marked reduction in the body weights and individual organ weights of their pups when compared to well-nourished pups. At weaning, the body and organ weights of pups handled during the nursing period were greater than those of pups not handled during the nursing period. This difference in weight of handled animals disappeared by 55 days of age in the well-nourished animals but was still apparent at 55 days of age in poorly nourished animals. Finally, when analyzed with respect to body weights, the rank order of weights of all three brain sections measured was NM $\geq$ HM $\geq$ HW $\geq$ NW and that of adrenal weights with respect to body weights was HW $\geq$ NM $\geq$ HM $\geq$ NW.

Nucleic Acid Measures

Table 10a shows the results of the nucleic acid determinations of the forebrains at 55 days of age. The amount of DNA in the forebrains of animals nursed by well-nourished dams was significantly greater than those nursed by malnourished dams (Table 10b).

Although the amount of DNA in the forebrains of those animals that had not been handled during the first three weeks of life was greater than in the forebrains of those animals that had been handled for each nutritional group (Table 10a), the difference was not statistically significant (Table 10b).

The amount of acid soluble nucleotides, RNA, and protein in the forebrains of those animals that had nursed from dams fed high protein diets were greater than the amounts of these constituents in the forebrains of those animals that had nursed from dams fed low protein diets regardless of whether they had been handled. On all three measures, the effects of differential nutrition were statistically significant with the well nourished animals having greater quantities of acid soluble nucleotides, RNA, and protein (Table 10b). When each well-nourished group was compared to its respective malnourished group, the only significant difference was that the NW animals had greater amounts of acid soluble nucleotides in their forebrains than did the NM group. With the exception of the acid soluble nucleotides in the well-nourished groups, there were more acid soluble nucleotides, RNA and protein in the forebrains of those animals that had been handled during early life than in the forebrains of those

Table 10a

Nucleic Acid Measures

		Handled Malnourished	Non-handled Malnourished	Handled Well Nourished	Non-handled Well Nourished
DNA / Brain (mg)	$\bar{X}$	0.98	1.00	1.05	1.10
	s.d.	0.10	0.16	1.03	0.22
Acid Soluble Pool / Brain (mg)	$\bar{X}$	2.01	1.86	2.05	2.08
	s.d.	0.20	0.17	0.23	0.95
Acid Soluble Pool / Cell (mg)	$\bar{X}$	2.15	1.87	1.97	1.96
	s.d.	0.34	0.31	0.28	0.31
RNA / Brain (mg)	$\bar{X}$	1.95	1.79	2.03	1.99
	s.d.	0.17	0.25	0.14	0.18
RNA / Cell (mg)	$\bar{X}$	2.00	1.83	1.95	1.85
	s.d.	0.19	0.23	0.18	0.24
Protein / Brain (mg)	$\bar{X}$	77.48	75.45	83.27	78.67
	s.d.	8.16	8.62	5.63	8.08
Protein / Cell	$\bar{X}$	79.48	74.56	79.89	73.23
	s.d.	9.34	8.23	10.05	8.28

Table 10b

ANOVAR for Nucleic Acid Measures

	Source Variation	d.f.	Sums of Squares	Mean Square	F	P
DNA/Brain	Handling	1	0.0176	0.0176	1.00	NS
	Nutrition	1	0.106	0.106	5.50	< 0.025
	Interact.	1	0.00136	0.00136	0.00	NS
	Error	60	1.418	0.0236		
Acid Soluble Pool/Brain	Handling	1	0.0540	0.0540	1.51	NS
	Nutrition	1	0.245	0.245	6.88	< 0.01
	Interact.	1	0.108	0.108	3.04	NS
	Error	60	2.135	0.0356		
Acid Soluble Pool/Cell	Handling	1	0.302	0.302	3.30	NS
	Nutrition	1	0.242	0.242	0.23	NS
	Interact.	1	0.269	0.269	3.00	NS
	Error	60	5.046	0.952		
RNA/Brain	Handling	1	0.143	0.143	3.98	< 0.05
	Nutrition	1	0.281	0.281	7.84	< 0.01
	Interact.	1	0.050	0.050	1.40	NS
	Error	60	2.149	0.036		
RNA/Cell	Handling	1	0.264	0.264	5.97	< 0.005
	Nutrition	1	0.00157	0.00157	0.03	NS
	Interact.	1	0.0173	0.173	0.39	NS
	Error	60	2.65	0.0442		
Protein/Brain	Handling	1	157.21	157.21	2.69	NS
	Nutrition	1	290.38	290.38	4.97	< 0.05
	Interact.	1	23.46	23.46	0.40	NS
	Error	60	3502.74	58.38		
Protein/Cell	Handling	1	497.55	497.55	5.90	< 0.025
	Nutrition	1	2.99	2.99	0.03	NS
	Interact.	1	10.82	10.82	0.13	NS
	Error	60	4872.54	81.21		

animals that had not been handled for each nutritional group (Table 10a). However, only the amount of RNA was significantly greater in the forebrains of those animals that had been handled than in the forebrains of those animals that had not been handled (Table 10b), and there were no statistically significant differences between any of the handled animals and the corresponding non-handled animals (Table 10c).

Table 10c

Post Hoc Comparisons for Nucleic Acid Measures

	HW vs NW	NM vs NW	HM vs NM	HW vs HM
DNA/Brain	NS	NS	NS	NS
Acid Soluble Pool/Brain	NS	< 0.05	NS	NS
Acid Soluble Pool/Cell	NS	NS	NS	NS
RNA/Brain	NS	NS	NS	NS
RNA/Cell	NS	NS	NS	NS
Protein/Brain	NS	NS	NS	NS
Protein/Cell	NS	NS	NS	NS

The effect of the handling and dietary manipulations on the acid soluble nucleotides, RNA, and protein per cell in the forebrains

are also presented in Table 10a.[1] The amount of acid soluble nucleotides that had not been incorporated into RNA or DNA was greatest in the cells of those animals that had been given daily handling and nursed by dams fed low protein diets, and least in the cells of those animals that had not been given daily handling but had been nursed by dams fed low protein diets (Table 10a). However, none of the differences among the four groups were statistically significant (Table 10b) and the amounts of RNA and protein in the cells of the four groups of animals were similar. There were no differences between the well-nourished and malnourished animals in either the amount of RNA per cell or protein per cell for either of the two handling conditions (Table 10b). However, there were greater amounts of both RNA and protein in the cells of both groups of handled animals than in the cells of both groups of non-handled

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1. Since DNA is found primarily within the nucleus of a cell, and since the amount present does not vary within a species from diploid nucleus to diploid nucleus, it is possible to calculate the number of cells in a given organ. (Enesco and Leblond, 1962, in Winick and Noble, 1965). The formula is:

$$\text{Number of nuclei (millions)} = \frac{\text{Total organ DNA (mg)} \times 10^3}{6.2}$$

The denominator (6.2) is the amount of DNA (Mcg) in one diploid rat nucleus. (Enesco, 1957, in Winick and Noble, 1965). Using this figure, it is further possible to calculate RNA or protein per cell as follows:

$$\text{RNA (or protein) / nucleus} = \frac{\text{Total organ RNA (or protein} \times 10^3)}{\text{Number of nuclei (millions)}}$$

animals (Table 10b) but the difference between each of the handled and the corresponding non-handled animals was not statistically significant (Table 10c).

Summary. Protein restriction to lactating dams and handling of the pups also altered the neurochemical composition of their pups' brains. The number of cells per organ was lower in those animals that had nursed from low protein dams, and these animals consequently had smaller forebrains than those that had nursed from high protein dams. However, the amount of RNA and protein in the cells of these animals' forebrains was unchanged as a result of early protein restriction when measured at 55 days of age. Finally, there were fewer cells in the forebrains of animals that had been handled in early life than in the forebrains of those that had not been handled. However, the amount of RNA and protein in the forebrain cells of these handled animals was greater than that in the forebrain cells of the non-handled animals for both the well nourished and malnourished animals.

DISCUSSION

In the present investigation, the effects of both malnutrition and daily handling of rats during the nursing period on the course of development of several open field behaviors as well as on a variety of somatometric and neurochemical measures were examined. A 2 X 2 design was utilized so that the effect of early handling could be examined in both well-nourished and malnourished animals.

Rat pups that were handled from the day of birth to 21 days of age made significantly more entries into the peripheral as well as the central squares of the open field, significantly more supported as well as unsupported vertical rears, significantly more entries into a square in which a rubber ball was suspended, and spent significantly more time manipulating the ball at 22, 23, 29, 36, and 50 days of age than rats not given such handling. The occurrence of a significantly greater number of central square entries at 91 days of age, a significantly greater number of supported vertical rears at 90 days of age, and of unsupported vertical rears at both 90 and 91 days of age as well as a significantly greater number of entries into the novel square at 90 days of age by handled than by non-handled animals when observations were first carried out at 90 days of age attested to the long-term consequences of early handling on the amount of open field activity in rats.

When analyzed without respect to handling, restriction of protein in the diets of lactating dams resulted in a short-term reduction in the amount of activity in the open field of their offspring. This was evidenced by the finding that rats nursed by dams fed a high protein diet entered significantly more peripheral and central squares than rats nursed by dams fed a low protein diet at 22 days of age but not thereafter. The short-term reduction in the open field activity of offspring of protein-restricted dams was also evidenced by the finding of a significantly greater number of entries into a square in the open field in which a rubber ball was

suspended at 22 days of age but not thereafter by rats nursed by dams fed a high protein diet than by rats nursed by dams fed a low protein diet. The only non-transitory effect of the differential early nutrition was in the amount of time spent manipulating the rubber ball. Animals that had been nursed by well-nourished dams spent significantly more time pawing and chewing the ball than animals that had been nursed by malnourished dams at 22, 23, 50, and 91 days of age.

In general, the difference between the open field activity of the HM animals and the NM animals increased with age. On every measure of open field activity with only one minor exception, the HM animals were more active than the NM animals whether they were tested in the early postweaning period or in adulthood. The HM animals were significantly more active than the NM animals in the central squares at 22 days of age, made significantly more supported vertical rears at 29, 36, and 90 days of age, significantly more unsupported vertical rears at 90 and 91 days of age, and entered the novel square significantly more times at 29 days of age. In contrast, the difference between open field activity of the HM animals and that of the HW animals decreased with age. The HW animals demonstrated greater amounts of open field activity than the HM animals at nearly every age, although with the exception of the amount of time spent manipulating the novel object, the difference between these two groups was only statistically significant on the first test at 22 days of age.

The impetus for this investigation came from a suggestion by Frankova (1968), who, after observing the behavioral consequences of early-handled versus non-handled animals and of early well-nourished versus malnourished animals, questioned whether it would be possible to partially compensate for the developmental retardation that results from early malnutrition by providing extra handling at the same time as the malnutrition. This question formed one of the basic hypotheses of the present study - namely - that rat pups malnourished during the nursing period who are simultaneously handled would have greater activity levels in the open field than malnourished animals who are not provided this extra stimulation. This hypothesis was supported by the findings of the current experiment. Daily handling during the first three weeks of post-natal life resulted in an increase in the amount of activity in the open field both during the four weeks immediately after weaning and 10 weeks after weaning.

Although both the HW and HM animals demonstrated greater amounts of all open field activities than the corresponding groups of non-handled animals, as predicted, there was one major finding of the current experiment which was unexpected. In most of the open field measures taken at the younger ages, the rank order of groups was: HW $\geq$ HM $\geq$ NM $\geq$ NW. In fact, with the exception of peripheral square activity at 29, 36, and 50 days of age, central square activity at 36 days of age, supported vertical activity at 50 days of age, and the amount of time spent manipulating the novel

object at 36 days of age, the HW animals were significantly more active than the NW animals. Thus, while handling resulted in greater open field activity in the short-term regardless of the early nutritional status, the effects of handling were far greater in those rats that had been nursed by dams fed high protein diets than in those rats that had been nursed by dams fed low protein diets.

The rank order of most of the open field measures monitored in adulthood was HM $\geq$ HW $\geq$ NW $\geq$ NM with the handled groups demonstrating significantly more supported and unsupported vertical activity and novel square entries at 90 days of age, and significantly more central square activity and unsupported vertical rearing at 91 days of age. Only at 91 days of age, and only for the amount of time spent manipulating the novel object did the well-nourished animals demonstrate a significantly greater amount of activity than the malnourished animals. The relationship between the horizontal and vertical activity of the NW and the NM animals at 90 and 91 days of age was also the same as that reported by Franková and Barnes (1968a) for animals tested at 75 and 85 days of age.

The basic similarities among the results of most of the open field behaviors monitored suggests that they are all equivalently effective as measures of the consequences of early handling and early malnutrition on activity in an unfamiliar environment. The amount of activity in the open field was originally used by

Hall (1934, 1936) to measure differences in emotionality. According to Hall, the more emotional animals would move less and defecate more than less emotional animals. More recently, Denenberg (1969) supported Hall's contention with an analogy to the natural environment. He reasoned that some degree of inactivity typically occurred when the animal is confronted by a predator (a noxious stimulus), rendering the predator less perceivable. Therefore, the amount of time spent immobile, or conversely, the amount of activity an animal engaged in, would be a function of the animal's interpretation of the environmental threat. According to Denenberg, this was a measure of the animal's level of "emotionality". Other studies tend to support Denenberg's argument. Candland and Nagy, (1969), and Levine, Haltmeyer, Karas, and Denenberg, (1967) have reported negative correlations between physiological measures known to reflect stress, (e.g., plasma steroid concentration) and open field activity or amount of defecation in the open field. Although physiological measures to document freezing behavior were not taken in the present study, the earlier findings are relevant to the qualitative findings of the present experiment, i.e., greater amount of inactivity in non-handled than in handled animals. This may mean, according to Denenberg (1969), that the non-handled animals, not having been exposed to the range of stimulation and the stress concommitant with early handling, were less prepared later on to cope with the unfamiliar stimuli of the open field. Considering Levitsky's

(1971) hypothesis that malnutrition may simulate the non-handled condition, these speculations may also be relevant to the lesser activity of both groups of malnourished animals when compared to the handled well-nourished animals.

The behavior most commonly measured in the open field has been the amount of horizontal locomotion.[1] The present experiment differentiated between horizontal locomotion in the central squares and horizontal locomotion in the peripheral squares. Since the results obtained for the two measures were essentially the same,

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1. In spite of this experimenter's belief that the six open field measures that were monitored are all reflections of the same phenomenon, they will be discussed separately where necessary in order to compare and contrast the results of this experiment with those of others. It should, however, be noted that it is difficult to make comparisons among different open field studies. In the case of the nutritional investigations, all except that of Franková (1968) manipulated the independent variable either in a different manner, or for a different period or duration than the present experiment. They also tested at different ages, and at various times following nutritional rehabilitation. In addition, no information is available concerning how strictly environmental controls were maintained. In the case of the investigations of early handling effects, the animals remained outside their cages for varying lengths of time and were actually manipulated for different periods and tested at different ages and different times following the handling. Finally, no attempts have been made to standardize other variables which could influence open field performance such as strain differences, the dimensions of the field, the length of the testing sessions, whether testing was sequential or only occurred once, the ambient light and temperature in the experimental chamber, the manner in which the animals entered the field, the time of day of open field observations, what point in the animals' light-dark cycle observations occurred, and whether observations were under the same or different lighting conditions as home cage lighting.

they will be discussed together. During testing of the younger animals, the rank order of the four groups in horizontal locomotion was HW $\geq$ HM $\geq$ NM $\geq$ NW with minor reversals in the peripheral square activity of the NM and NW animals at 29 and 36 days of age. The results of the Franková and Barnes (1968a) study[1] can be compared to those of the present study. Although the data of neither the present study nor that of Franková and Barnes reached the conventional level of statistical significance, the direction of the difference between the amount of horizontal activity of the NM animals and that of the NW animals was the same in both studies. In addition, Franková and Barnes (1968a) measured the duration of inactivity and the percentage of animals that were inactive in the open field. They reported that 100% of their NM animals engaged in long periods of inactivity ($\bar{X}$ = 48.7 seconds) while only 33% of their NW animals engaged in short periods of inactivity ($\bar{X}$ = 5.3 seconds). These findings are consistent with the present finding that the NM animals' horizontal activity consisted largely of rapid bursts of activity interspersed with periods of inactivity while the NW animals generally maintained a slow and steady

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1. With the exception of the Levitsky and Barnes (1972) and Franková (1968) studies, all studies have either manipulated the nutrition variable or they have manipulated the handling variable. It is therefore assumed that the animals in those studies in which nutrition was manipulated were "non-handled", and the animals in those studies in which handling was manipulated were adequately nourished.

locomotion. These findings make it important to consider the contribution of length and frequency of inactivity when comparing amount of inactivity between groups. Levitsky and Barnes (1969) extended the period of malnutrition to seven weeks of age, and tested rats in the open field following six weeks of nutritional rehabilitation. They similarly reported greater horizontal activity among malnourished than control-fed animals. In a subsequent study (1972) these same investigators also manipulated early diet and handling simultaneously, as was done in the present study. With a seven week treatment period, and testing following 10 weeks of dietary rehabilitation, the relative amounts of locomotor activity demonstrated by the two groups of animals that were comparable to the non-handled animals and the two groups of animals that were comparable to the well nourished animals of the present study were the same as those found in the present study.

Finally, the only study to separately measure locomotor activity in the central squares and locomotor activity in the peripheral squares reported that the pups of gestationally-restricted dams spent significantly less time than controls in the central squares. (Simonson et al., 1971). It is interesting to note that similar results were obtained in the present experiment with lactationally-restricted pups.

Many other studies (e.g., Denenberg, 1969; Levine et al, 1967) have reported that early-handled animals engage in more horizontal locomotor activity than do their non-handled counterparts. These

results, and their relation to physiological findings (Candland et al, 1969; Levine et al, 1967) raise two questions. First, handled animals have been reported to perform "superiorly" to non-handled animals on a variety of tasks (Bernstein, 1957; Denenberg and Karas, 1960; Levine, 1956). The possibility may be entertained that these differences in performance are merely a reflection of basic differences in activity levels. This possibility is not contradicted by the greater-than-control amounts of locomotion demonstrated by malnourished animals which perform "inferiorly" on these same tasks. Differences in the quality of the increased activity levels in the handled animals (maintained levels of activity) and the NM animals (alternating periods of running and inactovity) may account for the better performances in one case, and the poorer performances in the other. Second, in the present experiment, the handled animals of both nutritional groups demonstrated more locomotor activity than the non-handled animals. However, the difference between the amount of behavior of the HW animals and their non-handled counterparts was far greater than the difference between the HM animals and their non-handled counterparts. This raises the question of the extent to which early handling can overcome the negative effects of early nutritional restriction.

One possible explanation for these open field results comes from Frankova's (1971) systematic observations of the relationship between nutrition during the lactation period and maternal behavior of rats. She reported that dams fed low protein diets (12% casein)

took longer to retrieve scattered pups, retrieved fewer pups during observation periods, and spent more time in extra-maternal behaviors than did control-fed dams. Two inferences may be drawn from these observations. First, in addition to the reduced quantity of milk available to the pups of low protein dams, the mothers may have engaged in nursing on fewer occasions or for shorter periods of time. This would accentuate the nutritional deprivation of the pups in this study or in any study where the lactating dams were nutritionally deficient. Second, the lesser degree of maternal-pup interaction in the low-protein litters suggests a simulation or accentuation of the understimulated condition and may have implications for the later behavior of these animals. The small amounts of novel square activity of the non-handled and malnourished animals in the present study suggests that external stimulation may also be minimal in these animals even beyond the litter period. In other words, the ultimate effects of the low levels of early stimulation may be accentuated by the later self-initiated avoidance of further stimulation. These effects would be maintained in the NM animals, but would be attenuated in those which were handled coincidentally with the deficient dietary intake (HM). Even if both the NM and the HM animals had similar intralitter stimulation during the first three weeks of life, the HM animals may have compensated for this in part by their higher levels of self-initiated interactions with the environment. These observations lend support to Frankova's (1968) earlier suggestion on which the present experiment was

based - - - that the addition of external stimulation (handling) might compensate for the behavioral deficits of malnourished animals. Early handling of malnourished animals may provide a partial replacement of some of the stimulation that would normally be present in the well-nourished animal at this time.

Another possible explanation for the greater amount of open field activity in the handled than the non-handled animals derives from the handling procedure utilized. Since each pup in the handled group was placed in a large metal container for 20 minutes and individually stroked in the experimenter's hand for two of these minutes, additional tactile, visual, olfactory, proprioceptive and motor stimulation opportunities were available. Such experiences prior to exposure to the open field could have reduced the unfamiliarity of the actual open field for the early-handled animals and resulted in a greater amount of activity both initially as well as later on. To control for this possibility, future studies would either have to minimize the similarities between the open field and the consequences of the handling procedure, or else offer both the handled and non-handled animals the same prior experience.

In the present study, the number of squares entered by all four experimental groups decreased from the first test at 22 days of age to the second test at 23 days of age and increased at successive tests thereafter to 50 days of age, except for a decline between 36 and 50 days of age in the NW group. The decrease in amount of activity from the first exposure to the open field to

the following day is probably a transient change - either of habituation or adaptation. This finding is in agreement with that of Denenberg (1969) who reported a decrease in open field activity from the first to the second day of testing in the open field in a group of unhandled animals. The longer term changes, on the other hand, probably represent a true developmental phenomenon. Despite differences among the groups, an overall similar pattern of increased activity with age could be accounted for by the fact that the animals grew in size while the squares of the open field remained the same size. The increase in activity level with increase in age is in keeping with age changes reported in other studies. Candland and Campbell (1962) studied locomotion during a ten minute period in the open field at 18, 23, 30, 44, 54, 65, and 200 days of age with a different set of animals being tested at each age. As was the case in the present study, they found that older animals were more active than younger animals. They also found that rats were slightly less active between 50 and 65 days of age than at other ages. Similarly, Jewett and Norton (1964) reported that both rats raised in isolation and in groups of four to five had peak open field activity levels at 35 days of age with asymptotes thereafter. A possible explanation for the decline in activity at 50 days of age in the present study is that it may have been the result of three shorter intervals between tests followed by a longer interval, making rehabituation to the open field necessary. In the Candland and Campbell study, however, each test was carried out with a different group of rats. Therefore, the

increase in activity from 22 to 36 days of age and the asymptote to 50 days of age in the current investigation is probably a developmental phenomenon rather than an artifact of repeated testing.

Many of the trends that were apparent in the locomotor activity in the peripheral and central squares were also apparent in the supported and unsupported vertical rearing measures. Supported vertical rearing necessarily occurred in the peripheral squares while unsupported vertical rearing could have occurred in the central or in the peripheral squares. The rank order of the groups in amount of horizontal peripheral square activity was the same as that for the vertical supported activity in the early-tested animals (HW $\geq$ HM $\geq$ NM $\geq$ NW), including the position reversals of the two non-handled groups at 29 and 36 days of age. In contrast, the group differences in central squares activity only grossly corresponded to the differences between the groups in amount of unsupported vertical activity. It may be possible to account for this by the fact that whereas supported vertical activity was necessarily carried out in the peripheral squares, unsupported vertical activity could have been carried out anywhere in the field.

Results similar to those of the present study have been reported by other studies that have measured amount of vertical rearing in the open field. Frankovà and Barnes (1968a) reported a greater number and duration of standing-up-reactions in rats that had been malnourished for the first seven weeks of life when compared to control-fed rats. This could be compared to the greater number of vertical rears

in the NM animals than the NW animals of the present study. In another study, Frankovâ (1968) obtained results that were similar to those of the present study: Both well- and poorly-nourished animals[1] that had been handled in early life[2] demonstrated more standing-up-reactions than corresponding non-handled animals when they were tested at 90 and 110 days of age. Frankovâ found the greatest number of standing-up-reactions in the intermediate sized litters (nine or 13 pups per lactating dam) that had not been handled, and the least in the largest litters (17 pups per lactating dam) that had not been handled. This would be comparable to the greater number of vertical rears in the HW or HM animals than the NM animals of the present experiment.

In the current experiment, the rank order of the four groups in the number of entries into the novel square and the amount of time spent manipulating the ball was the same as that for the other open field behaviors measured. The difference among groups, however, were more marked. This suggests that novel square activity is a more sensitive measure of the effects of early handling and early malnutrition. The probable explanation is that the two groups of non-handled animals often reversed their direction of locomotion to avoid entering the square; or, when they did enter the square, instead of engaging the ball with their teeth or paws, they often remained

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1. Early dietary intake was varied by manipulating litter-size.

2. There were two uncontrolled variables in this study: 1) the intralitter stimulation resulting from the manner of dietary manipulation, and 2) the precise nutritive status of individual pups when there was competition for teats.

immobile behind the ball. In contrast, the two groups of handled animals often vigorously pawed and chewed the rubber ball and repeatedly engaged it between their teeth, walked with it to another corner of the open field, released it, and visually followed its bounce against the chamber walls. It is apparent from these observations that animals that had had extra handling during the nursing period were likely to investigate this particular unfamiliar stimulus whereas, animals that had not had such handling avoided, or at least ignored this stimulus. The data of the current experiment do not provide an answer to the question of why early handling produces an animal engaging in investigatory activity in an unfamiliar environment.

The HM animals demonstrated the most profound increase in the number of entries into the novel square from 22 to 36 days of age. In contrast, the HW animals, already entering the novel square a great number of times at 22 days of age, demonstrated the smallest increase in this behavior from the first to the fourth test session. It is possible that the HW animals began at near asymptotic levels and could not increase this behavior while continuing to engage in other behaviors as well. Another explanation of the failure to increase the number of novel square entries during infancy in the HW group may derive from the fact that at successive ages, each entry into the square by the HW animals was accompanied by longer periods of attending to the ball which would make the animal less likely to immediately re-enter the square. On the other hand, the marked

increase in the novel-square entry of the HM animals from the first test at 22 days of age to the fourth test at 36 days of age may reflect the effects of handling. More specifically, it may be an example of how handling can partially compensate for the effects of nutritional deficit in behaviors that permit exploration of the environment. The possibility that malnutrition exerted a short-term effect which was overcome following dietary rehabilitation may be ruled out because the NM animals did not increase their novel square entries from 22 to 36 days of age.

A number of other studies have investigated the response of suboptimally nourished animals to novel stimulation. Levitsky and Barnes (1970) reported that rats malnourished for the first seven weeks of life demonstrated significantly greater suppression of open field activity following the introduction of a loud buzzer sound than did control-fed rats. In a subsequent study, (1972) they also found that early malnourished animals entered a chamber adjacent to an open field fewer times than did well-nourished animals, especially if they had not received extra stimulation during early postnatal life. Barnes et al. (1970) reported that protein-deficient pigs exhibited more squeaking when placed in unfamiliar surroundings, and demonstrated more resistance to being harnessed than did control-fed pigs. Finally, Zimmerman, Strobel & Maguire (1970), studying protein-malnourished monkeys, reported that they avoided or did not approach unfamiliar objects. They termed this a "neophobic response".

On the one hand, a certain amount of timidity to an unfamiliar stimulus is probably an adaptive mode of responding. On the other hand, generalized fear in a wide variety of situations is probably an impediment to making possibly beneficial stimulation available to the organism. It could be hypothesized from the results of the present as well as previous studies that animals that are poorly nourished as well as not handled during nursing lack the ability to discriminate stimuli that should be avoided from those that may be approached. It could be further hypothesized that these groups of animals therefore have lower thresholds to stimuli which they interpret as threatening, and respond to with avoidance. This could impede the typical developmental pattern of gradual investigation and learning about critical aspects of the environment. To explore this possibility, future studies could utilize a design and independent variables similar to those of the present study, and also measure baseline activity in the home cage, and test for such investigatory behaviors as latency of emergence from the home cage, entry into auxiliary chambers in an open field, as well as response thresholds to various stimuli. This could provide some measure of whether suboptimally nourished animals that are not handled do in fact respond with avoidance more often than controls or whether differences in performance can be explained by differences in baseline activity. The ultimate significance of this manner of responding is obvious: stimuli that are avoided or are not responded to cannot be learned about nor can they be utilized as a basis for learning about other stimuli.

Another suggestion for future studies of open field behavior is that not only the amount of activity, but also the amount, duration, and nature of inactivity should be examined. This would make it possible to verify the observations of the present study which suggest that some of the differences in amount of activity were in fact due to differences in the amount of inactivity. In addition, testing should be carried out regularly from infancy to adulthood. This would make available to scrutiny whether the first introduction to an unfamiliar environment in adulthood would elicit different patterns of behavior than repeated reintroductions to that same environment during development. In the attempt to determine why handling and malnutrition affect open field behavior, future studies should also employ other behavioral measures including simple and complex mazes, passive and active avoidance tasks, and sensory discrimination tasks in addition to open field observations to see whether those tasks that are affected by early handling and malnutrition share certain features and whether there are differential effects on simple and complex tasks.

Somatometric

The most well documented finding in the study of animals that have existed on a less than optimum dietary intake during early life is their failure to gain weight relative to optimally nourished animals. The nutritional status of the animals in the present study was manipulated via a reduction in the protein intake of the lactating dam. One of the primary effects of suboptimal protein intake

is a decline in milk output without a concommitant change in nutrient quality or the relative amounts of all nutrients[1] (Meuller and Cox, 1946; Venkatachalam and Ram, 1964). Suboptimal protein intake also results in a decrease in the malnourished dams' body weights during the period of lactation while control dams gain weight during the same period. A secondary effect of low protein intake by a lactating dam is a proportional deficit in the intake of all nutrients by the nursing pups. Suboptimal weight gain in such pups was found in the present study as well as in numerous other studies (reviewed in Platt and Stewart, 1971).

The body weights of the pups were significantly greater in litters where the pups had been handled than in litters where the pups had not been handled. This effect of handling on the pups' body weight has been reported by several investigators working in other laboratories (Denenberg and Karas, 1959, 1960; Levine and Otis, 1958). Altman (1968) however, reported greater body weights for previously handled than non-handled rats at 41 days of age, but no differences at 11 and 14 days of age. Obviously, further study is necessary before the nature of the relationship between weight gain and handling and between weight gain and early nutrition is fully understood.

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1. At a critical low level of protein intake in a lactating dam, milk production would be completely curtailed.

Only one study investigated the effects of early handling on pup body weight in suboptimally nourished animals. Levitsky and Barnes (1972) found no weight differences between handled and non-handled animals in either nutritional group from birth to 24 weeks of age. In the present study it was found that the weights of the handled animals of both nutritional groups at weaning were higher than those of their non-handled counterparts. This weight difference was greater in those litters that had nursed from high-protein dams than in those litters that had nursed from low-protein dams. The HW animals were significantly heavier than the NW animals while the HM animals were not significantly heavier than the NM animals at weaning. While the explanation for the relative weight deficits in the nutritionally deficient dams and their pups is obvious, the explanation for the greater weights in the handled than in the non-handled pups is less self-evident. It is possible that an increase in a dam's food consumption could have occurred while her pups were absent from the cage for daily handling. This could result in an increase in her supply of milk and ultimately in the weights of her pups. It is also possible that a stress-induced hypermetabolism of the pups resulted from daily handling. This could lead, especially in those animals nursing from well-nourished dams, to a greater nutritional demand by the handled than by the non-handled animals, and consequently a more rapid weight gain. Bovard (1958) hypothesized that the effects of early handling are mediated by a permanent change in hypothalamic functioning. He considered this altered hypothalamic

functioning to be a secondary result of altered amygdaloid activity, which was the direct result of the early sensory input of handling. Lastly, he suggested that this hypothalamic change could result in elevated levels of growth hormone output. Although there is no empirical support for this hypothesis, it is a possible explanation of the current finding.

Another possible explanation for the weight differences between the handled and non-handled animals is in terms of a change in the mother-pup interaction consequent upon the pups' return to the cage following daily handling. The mother may have retrieved the pups more vigorously, or the pups could have suckled more vigorously upon returning to the cage. In Frankovà's (1971) study of the relationship between specific maternal behaviors (retrieval latency, and number of pups retrieved) and offspring weight in malnourished litters, she reported positive correlations between the weights of the pups and the mean number that were retrieved during an observation period, and negative correlations between the weights of the pups and the latency to retrieval of the first one. The possibility that the maternal-pup interaction may be altered following handling receives some support from the study of Barnett and Burn (1967) who reported that mouse pups which were either earpunched on day six, or removed from the nest for five minutes on days six through ten received three times as much contact from the dams upon being returned to the cage as those that were left undisturbed. If the low levels of intralitter stimulation in the low-protein litters does in fact contribute to the relative

deficiency of milk available to the nursing pups, and their consequent failure to gain weight, it is possible that the additional intralitter stimulation following handling may contribute to the partial weight compensation in this group of animals and perhaps also to the behavioral findings (i.e., HM $\geq$ NM).

The relative weights among the four groups of animals were somewhat different at 55 days of age from what they had been at weaning. Although the NM animals remained lighter in weight than the HM animals, the NW and HW animals reversed their relative positions. A possible explanation for this last result can be inferred from the results of the open field tests in which the HW animals were significantly more active than the NW animals. It is possible that these animals were also more active in their cages and therefore had a greater expenditure of energy relative to their caloric intake than did the non-handled animals. Since neither group would have engaged in a considerable degree of locomotor activity prior to 21 days of age, such an input-output energy function would have had no measurable effect on the 21 day body weights. The fact that the malnourished animals maintained their relative handled to non-handled weights from 21 to 55 days of age does not contradict this possibility. The differences between the HM and NM animals' activity levels in the open field were not as great as the differences between the activity levels of their well-nourished counterparts. Therefore, the energy expenditure: energy input ratio would not be expected to be reflected in a relative weight gain. In order to test this possibility, it would be

necessary to measure daily caloric intake as well as output throughout the study. It would also be useful to observe activity in the home cages as well as activity in the open field at all ages. This would give an indication of whether the open field activity scores were a reflection of overall activity or whether they were a specific response to an unfamiliar environment.

The absolute weights of the forebrains of the four groups of animals in the present study varied with body weights. Both nursing from high-protein dams and early handling resulted in significantly greater forebrain weights than nursing from low-protein dams and no handling. In addition, the forebrains of both groups of well-nourished animals were significantly heavier than those of the corresponding groups of malnourished animals. Other investigations have found similar effects on forebrain of animals subjected to suboptimal diets during early life, (reviewed by Platt and Stewart, 1971). In the present study, the forebrain weights of the handled animals of each nutritional group were slightly, but not significantly greater than the forebrain weights of the corresponding non-handled animals. Other studies (Tapp and Markowitz, 1963) have reported similar results for handled versus non-handled animals. Culley and Lineberger (1968) suggested that the absolute brain weights of nutritionally deficient animals may be commensurate with a younger chronological age rather than with the actual chronological age. Altman (1968), who found that the brain weights (as well as other developmental indices) of handled animals were lighter relative to non-handled animals at 11 and 14 days

of age but heavier (as in the present experiment) at 41 days of age, hypothesized that there was a deceleration of maturation in handled animals which was actually a prolongation of the period of development (termed "fetalization"), possibly permitting a longer period of environmental influence on the brain. Inspection of this phenomenon phylogenetically gives reason to suspect that the longer the period of postnatal development, the greater is the ultimate functional capacity of the organism (Altman, 1968). The implication is that the same is true ontogenetically for the handled organism. If the handled animals do in fact have a prolonged period of postnatal development, as Altman implied, this would mean that the time during which the environment is capable of exerting its most profound influence would also be prolonged. If Altman's explanation is valid, it would have important behavioral implications, especially for the handled malnourished animals. If the malnourished animals are prevented from reaching their genetic potential for neurological development and corresponding behavioral development, it might be possible that a prolongation of the period of development would act to re-establish attainment of this potential.

Perhaps a more meaningful way of analyzing forebrain weights is their relation to each animal's body weight. The results of the present experiment indicate that the brain weight : body weight ratios at 55 days of age of those rats that had been nursed by dams fed low protein diets were higher than those that had been nursed by dams fed low protein diets were higher than those that had been nursed by dams

fed high protein diets. This is a common finding in rats (Guthrie and Brown, 1968; Widdowson, Dickerson, and McCance, 1960) as well as in dogs (Platt and Stewart, 1968), pigs (Dickerson, Dobbing, and McCance, 1966; Platt, Pampiglione, and Stewart, 1965) and even in children (Brown, 1965). The elevated brain weight:body weight ratio of the low-protein subjects can be interpreted in at least two ways. First, a greater ratio of any organ weight to body weight in a sub-optimally nourished organism may be simply a function of the relative sparing of any non-fatty tissue over any tissue with a large proportion of fat. Second, Widdowson et al., (1960) suggested that the elevated brain weight:body weight ratio could be interpreted as an indication of central nervous system immaturity. If high brain weight:body weight ratios could be assumed to be a valid index of immaturity, then the NM animals of the present study fell at the lowest end of the spectrum, and the NW animals fell at the highest. However, the higher body weights at 55 days of age of the NW group does not support this hypothesis. Metabolic explanations for this excess of body weight and better criteria for central nervous system maturation must be established before conclusions concerning relative maturity could be drawn from these data. The brain weight:body weight ratio could possibly be a useful index of maturity to a critical level, beyond which it could be inappropriate.

In addition to forebrain weights, the present study investigated the effects of handling and nutrition on cerebellar and brain

stem weights. Similar trends among the four experimental groups were apparent in these measures as in forebrain. Although not statistically significant for the cerebelli, the cerebelli and brainstems of both groups of well-nourished animals were heavier than those of both groups of malnourished animals, and the weight of these two organs with respect to body weight were significantly greater in both groups of malnourished animals than in both groups of well nourished animals. Both Chase, Lindsley and O'Brien (1969) and Howard and Granoff (1968) studied cerebellum weights of undernourished and adequately nourished rats, and reported the same relationships as were found in the present study. The interpretation of these results would be the same as that for forebrain. In addition, speculations could be made concerning cerebellar size and the inferior motor functioning of poorly nourished children (Kahn, 1954), dogs (Platt and Stewart, 1969) and pigs (Platt et al., 1965).

The liver weights of the four groups of animals in the present study were a direct reflection of the body weights of these animals. The absolute liver weights of the well-nourished animals were significantly greater than those of the malnourished animals, but when liver weights were analyzed with respect to body weight, there were no significant differences among the four groups. The fact that there were differences among the four groups of animals when three different parts of the brain were analyzed with respect to body weight, but not when the liver was analyzed with respect to body weight is an indication that what has occurred in the brain is

specific to the central nervous system and is not a general physiological reaction to early malnutrition. Another study (Coombs, 1972) found that while RNA and protein per cell were similarly depressed by malnutrition in both brain and liver, there was no effect of handling on these measures in liver.

Neurochemical

The current finding of greater amounts of DNA in the brains of well-nourished animals than in the brains of malnourished animals indicates that there are more cells in the brains of the well-nourished than the malnourished animals. The present study also found greater amounts of the cellular constituents measured (acid soluble pool, RNA, and protein) in the brains of animals that had nursed from high protein dams than in the brains of animals that had nursed from low-protein dams. The HW animals had more acid soluble nucleotides per cell than the NM animals, but less than the HM subjects. The acid soluble pool is an index of the synthetic activity of nucleic acid precursors. In order to understand the meaning of this measure, the end products of nucleic acid catabolism would also have to be quantified. Rosso, Nelson and Winick (1971) did this in the brains of well-nourished and malnourished rats. They reported that RNase (a likely marker of RNA degradation) per cell was greater in the brains of lactationally-malnourished rats. Other investigators (Wannemacher, Wannemacher & Yatvin, 1970) reported an increased rate of RNA synthesis during malnutrition. The conclusion from these two studies is that mal-

nourished organisms may have fewer precursors available for incorporation into RNA, and both incorporation of these precursors and degradation take place at a more rapid rate in malnourished animals than in normals. Although the present study only measured the synthetic aspects of nucleic acids in the forebrain, it is apparent that this is greater in malnourished animals that are handled than in malnourished animals that are not handled in early life. It may be inferred from the amounts of RNA and total protein in these animals' brains that catabolism is not simultaneously increased. Considering the possible role played by RNA in learning (Rosenzweig, Krech, Bennett and Diamond, 1968), the implications of an increased availability of precursors without a concommitant degradation of RNA resulting from early handling are profound.

Both RNA and protein were significantly greater in the brains of those animals that had nursed from high-protein dams than in the brains of those animals that had nursed from low-protein dams. However, the amount of neither RNA nor protein per cell was different in these two groups. The meaning of these results is that DNA synthesis was curtailed by malnutrition during the critical phase for its occurrence, and subsequent adequate nutrition permitted the attainment of normal sized cells. But, the beginning of nutritional rehabilitation after the period of DNA synthesis prevented the development of the normal number of cells. These results are consistent with those reported by other experimenters (Fish and Winick, 1969a; Howard and Granoff, 1968; Winnick and Noble, 1965,

1966) for rats as well as for pigs (Dickerson et al, 1966; Platt et al, 1965) dogs (Platt and Stewart, 1969) and also for young children (Winick and Rosso, 1969b). The present demonstration of an effect of malnutrition on nucleic acids in the brain of rats at 55 days of age, when considered in conjunction with the findings of previous studies in which nucleic acid determinations were made at different ages, further supports the irreversible consequences of preweaning dietary deficiency on brain cell number. Although the difference was not statistically significant, there were fewer cells in the forebrains of those animals that had been handled in early life than in the brains of those that had not been for both nutritional groups. However, each of these cells contained significantly more RNA and protein than the cells in the forebrains of the non-handled animals.

Other studies that made nucleic acid determinations of the brains of handled versus non-handled animals (Coombs, 1972; Krylov and Kochegarova, 1975), reported results similar to those of the present one. Glassman (1969) postulated that certain types of early stimulation would result in an increased rate of RNA and protein synthesis in brain cells. He reported that 50% more uridine was incorporated into the brains of mice that had been trained to avoid electric shock than the brains of yoked controls. This, he said, was basically the result of increased synthesis, and decreased degradation of RNA. The report by Coombs (1972) of significantly lower levels of RNase in the brains of handled than non-handled controls suggests that Glassman's postulation may apply to handled animals as well.

The differences between the handled and non-handled animals of both nutritional groups were the same for acid soluble nucleotides, RNA, and protein per cell in forebrain in the present study. The handled animals had somewhat fewer, but far larger cells than the corresponding non-handled animals. Since we have said that the reduction of cell number in the nutritionally-restricted animals is most likely a permanent and a negative effect, it would be difficult to claim that the reduction of cell number in the handled animals is either a temporary, or a positive effect. However, the reduction in cell number in both sets of handled animals is accompanied by an increase in cell size. In fact, in the HM group, the size of the cells was nearly that of their well nourished counterparts. Further, these results are consistent with Altman's suggestion of an extension of the period of development ("fetalization").

At present it is impossible to attach functional significance to differential cell size or differential cell number. Far more study will be necessary to translate these empirical observations of neurochemistry into practical predictions about function. The rank order of the number of cells in the forebrains of the four groups was NW $\geq$ HW $\geq$ NM $\geq$ HM. There is no relationship between this order and the order obtained from the analysis of open field behaviors. However, the rank order of RNA in each cell of the forebrain was HW $\geq$ HM $\geq$ NW $\geq$ NM, and the rank order of protein in each cell in the forebrain was HW $\geq$ HM $\geq$ NM $\geq$ NW. These were

similar to the rank order of most pre-pubertal measures in the open field.

Relationship Between Early Nutritional Status and Early Handling

There is no information establishing that early nutrition and early handling operate through the same mechanism(s). Several experiments, however, suggest possible commonalities. One hypothesis comes from a series of studies first conducted by Schaefer, Weingarten and Towne (1962), and later supported by Schaefer (1963, 1968, 1971) as well as by Levine and Mullins (1968) and Hutchings (1963, 1967, 1968). In a series of experiments, Schaefer and his colleagues (Schaefer, 1968, 1971) studied both physiological responses (adrenal ascorbic acid depletion to cold stress) and behavioral responses (defecation and post-click crouching in the open field as well as suppression of lever pressing activity following a conditioned avoidance stimulus) of animals that had been 1) handled, allowing a drop in core temperature; 2) handled without allowing a drop in core temperature; 3) not handled, but with a drop in core temperature; or 4) not handled, and with no drop in core temperature. The results of these studies indicate that a crucial result of handling is the drop in core temperature that occurs when the animal is removed from its nest, and that handling itself is an ineffective stimulus without a coincident drop in temperature.

Hutchings (1967) later corroborated Schaefer's findings, with one addition. Hutchings exposed rats during their first week of life to temperatures that either resulted in rapid or moderate rates

of heat loss for varying periods of time and also resulted in different ultimate core temperatures. He reported that the essential factor in cold exposure was not the absolute loss in body temperature, but instead, the rate of temperature loss. He further reported curvilinearity in open field crouching behavior as a function of heat loss.

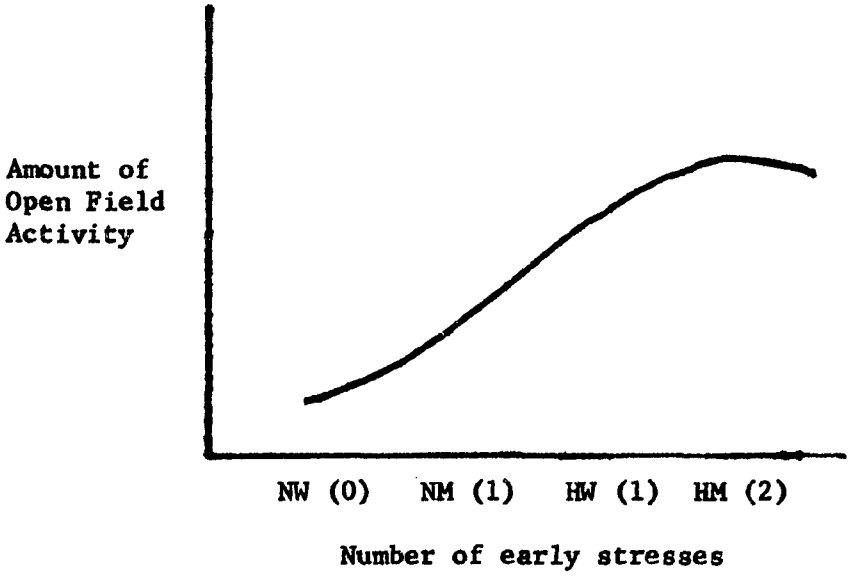
The results of these experiments strongly suggest that hypothermia does play a crucial role in the development of the "handled" animals. Schaefer (1971) suggested that immediate hormonal changes take place as a result of the drop in temperature. These changes alter ongoing biochemical processes which further modify cellular development. Ultimately, permanent changes can be produced by the effect of these changes on intermediary metabolism as well as their effect on tissues and organ systems. Levine and Mullins (1968) suggested that the steroid hormones which are present earlier and in greater quantities in early handled animals act directly on the brain to modify its ongoing development as well as the later expression of physiological and behavioral processes.

These observations on temperature change and susceptibility to early stimulation are interesting in light of the relation between nutritional status and body temperature. Heggeness (1962) studied temperature change as a function of pre-weaning nutritional status. He reported that lactationally-deprived rats were less efficient in maintaining core temperature and developed temperature regulatory capacity slower than did well-nourished (presumably

unhandled) controls. Similarly, Chow and Lee (1964) reported decreased resistance to hypothermia in rats that had been nutritionally deprived during the pre-weaning period. Finally, Brobeck (1945) suggested that the greater activity levels of nutritionally-deficient animals may be an attempt to compensate for their lesser body temperatures. This relationship between animals that are suboptimally nourished during an early period and animals that are handled or exposed to similar environmental manipulations during an early period is one that deserves further study. Animals could be malnourished while being maintained at supra-normal temperatures in order to prevent a drop in core temperature. The development of homeothermia as well as the development of activity levels in these animals could be compared to those of malnourished animals that had not been maintained at supranormal temperatures and well nourished controls. Knowledge of the physiological mechanism of both the suboptimal nutrition and the subnormal temperatures would also aid in the understanding of how it might be possible for one to exert an influence on an organism's response to the other.

Another possible explanation for the differential open field activity of the four groups of animals in the present study would be in terms of the relationship of nutritional status and handling to "stress" stimulation. The hypothermia resulting from early handling and from early nutritional deficiency may be considered to be stressors, subjecting the HM animals to a dual experimental stress, the NW animals to no experimental stresses, while the NM

animals and the HW animals were both subjected to a single experimental stress. Since the rank order of the amount of activity in the open field was, in general HW $\geq$ HM $\geq$ NM $\geq$ NW, the amount of open field behavior could be described as similar to a negatively accelerating function of the amount of early stress as follows:



Frankova's (1968) results could be interpreted in this same manner. She reported that while early handling resulted in greater open field behavior at 90 days of age in animals raised in litters of 4, 9, 13, and 17, this effect was most pronounced in the animals from litters of 9 and 13. If the non-handled animals are seen as the zero-stress situation and showed the least open field activity, handling may be seen as a single early stress, resulting in greater open field activity. Finally, handling plus rearing in a litter of 17 pups

as Franková did may be seen as a double early stress, and resulted in no more open field activity than did a single stress.

To further explain the relationship between open field activity and early stress stimulation, adrenal gland weight:body weight ratio was examined in the present experiment. The rank order of the four groups for this measure was HW $\geq$ NM $\geq$ HM $\geq$ NW. The difference between the adrenal gland weight:body weight ratio of the NW animals and the HW animals is consistent with the differences between comparable groups of animals reported by other investigators (Levine, 1957, 1962). Those animals that had the most obvious hypertrophy of the adrenal gland were the same animals that engaged in the most activity in the open field, and conversely, those animals that had the smallest adrenal gland weight:body weight ratios engaged in the least activity in the open field. In fact, except for the minor difference in the adrenal gland weight:body weight ratios of the two groups of malnourished animals[1], the rank order of the four groups in the amount of open field activity was the same as that for the adrenal gland weight:body weight ratio.

Various studies have reported early adrenocortical functioning (Haltmeyer, Denenberg, Thatcher, and Zarrow, 1966; Levine and Lewis, 1959), greater and more sustained plasma steroid levels following

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1. Since the actual size of the adrenal glands of the HM animals was larger than that of the NM animals, the greater adrenal gland weight with respect to body weight of the NM animals than the HM animals must be seen in the light of the greater body weights of the HM animals.

electric shock (Levine, 1962), and a lesser plasma steroid response following innocuous stimulation (Levine and Broadhurst, 1963) in rats that had been handled relative to rats that had not been handled. Further, Haltmeyer, Denenberg and Zarrow (1967) reported that rats that had been handled in infancy had greater plasma corticosterone levels immediately following electric shock at 75 days of age than rats that had not been handled in infancy, but lesser levels 15 minutes following the electric shock, indicating that the adrenal cortical response of rats that had been handled in infancy was specific to a stress stimulus, and not generalized for a period of time following the stimulus. Haltmeyer interpreted this to mean that handled animals had more adaptive responses to stress stimuli.

Considering the previous argument that the ability to explore an unfamiliar environment is a prerequisite to learning about that environment (i.e., an adaptive response), the weights of the adrenal glands in the four experimental groups of the present study are consistent with Haltmeyer's interpretation: Except for the relative positions of the two groups of malnourished animals, the amount of activity in the open field in early life was directly proportional to the adrenal gland weight:body weight ratio.

The implications of these observations are profound. It has been suggested (Schapiro, 1971) that the neonatal functioning of this hormone system exerts a priming effect on the central nervous system. Such an effect might have permanent hormonal as well as behavioral sequelae. In fact, Levine et al. (1967) reported that

animals that had been handled in infancy had lower steroid responses and greater horizontal activity in the open field at 80 days of age than animals that had not been handled in infancy. Since independent observations have reported greater open field activity (DeNelsky and Denenberg, 1967) as well as better performance on various learning tasks (Denenberg and Morton, 1962), similar relationships could probably be outlined for handled animals' steroid responding and performance on learning tasks.

Within the well-nourished group of the present study there is no reason to suspect a cause other than handling for the greater adrenal weight:body weight of the handled animals. If this is the case, then the optimal conditions for the development of behavior must be at least some amount of early stress (e.g., a protein deficient diet or early handling). The most important question here concerns the mechanism by which handling acts in the chow fed laboratory rodent to result in a better performance on certain tasks, and whether this same mechanism also acts in the malnourished rat to partially compensate for his already poorer performance on these same tasks. The results of the present experiment suggest an affirmative answer.

The period of life during which handling is effective has also been a subject of study. Schaefer (1971) reported that the effects of temperature change were only evident in those rats that had been subjected to extra handling during the first week of life - - - either the first week alone, or the first week in combination with the

second and third weeks. Others (Levine and Mullins, 1968; Denenberg, 1971) have also reported that handling or other experimental manipulations such as electric shock were only effective during some combination of the first weeks of life. In fact, newborn rats are not homeothermic (Hutchings, 1968). In the current investigation, it was observed that during the first seven to ten days of life, all pups, when removed from the cages for handling became cold to touch within the first ten minutes of removal from the cage. They also exhibited random movement until they contacted any warm object (another pup) when returned to the cage. This behavior ceased as the pups matured, grew hair, and probably developed sufficient metabolic thermogenesis to maintain body temperature. These later developments are probably coincident with the end of the period during which handling or cold exposure may result in permanent sequelae.

APPENDIX 1: Extraction of Nucleic acids and Protein

I. Tissue Removal

1. Rapidly remove tissues and weigh promptly.
2. Homogenize at 0-4°C and place in ice bucket.
3. Remove aliquot equal to approximately 250 mg wet weight.

II. Acid Precipitation and Wash

1. Add ½ homogenization volume of cold 0.6N perchloric acid (PCA) and mix.
2. Allow to stand 10 minutes in ice bucket.
3. Spin at 12,000 rpm for 10 minutes in the cold.
4. Discard supernatant.
5. Add 2-3cc. cold 0.2N PCA and mix.
6. Repeat steps 3 & 4.
7. Repeat steps 5, 3 & 4.

III. Lipid Extraction

1. Add 5cc. ice cold absolute ethanol containing 1% potassium acetate and mix.
2. Spin at 12,000 rpm for 10 minutes in the cold.
3. Discard supernatant.
4. If desired, samples can now be capped and frozen.
5. All subsequent lipid extraction steps are carried out at room temperature.
6. Add and mix, spin for 10 minutes and discard supernatant of the following solvents:
   a. 3cc. 80% ethanol
   b. 3cc. 3:1 ethanol:ether
   c. 3cc. ether
7. If desired, samples can now be capped and frozen.

IV. Removal of RNA

1. Add 4cc. of 0.3 KOH and mix.
2. Incubate in water bath at 37°C for 60 minutes.
3. Remove and add 2cc. cold 1.2N PCA and allow to stand 10-15 minutes in ice bucket.
4. Spin at 12,000 rpm for 10 minutes in the cold.
5. Save supernatant for RNA determination.
6. Add 2cc. cold 0.2N PCA and mix.
7. Repeat steps 4 & 5.
8. Repeat steps 6, 4 & 5.
9. Total supernatant fraction = RNA in 10cc. 0.2N PCA.

APPENDIX 1 (cont'd)

V. Optional Stopping Step

1. Add 3cc. cold absolute ethanol and mix.
2. Spin at 12,000 rpm for 10 minutes in the cold.
3. Discard supernatant.
4. Cap and freeze.

VI. Removal of DNA

1. Add 2cc. ice cold 1.6 PCA and mix.
2. Incubate in water bath at 70°C for 20 minutes with shaking.
3. Remove, place in ice bucket to cool.
4. Spin at 12,000 rpm for 10 minutes in the cold.
5. Save supernatant for DNA determination.
6. Repeat steps 1-5.
7. Total supernatant fraction = DNA in 4cc. 1.6N PCA.

VII. Protein

1. Add 3cc. 95% ethanol and mix.
2. Spin at 12,000 rpm for 10 minutes at room temperature.
3. Discard supernatant.
4. Add 5cc. 0.1N N_aOH and mix. Allow to stand at room temperature or at 37°C until pellet is dissolved.
5. Total supernatant fraction = protein in 5cc. 0.1N N_aOH.

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