

The Influence of Dietary Calcium on
the Development of Blood Pressure in
Neonatal Spontaneously Hypertensive Rats

by

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A THESIS

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The present study examined the influence of maternal dietary calcium on the electrolyte physiology and blood pressure of young spontaneously hypertensive rats (SHRs). Adult SHR dams were placed on low (0.1%), medium (1.0%), and high (2.0%) calcium diets. After 6 weeks on the diets, systolic blood pressure was taken and blood samples were obtained for assaying serum electrolytes. Two days later, the dams were bred. At birth, the offspring from normal calcium dams were fostered to mothers on the low, medium, and high calcium diet conditions. Blood samples were drawn from the pups at 16 and at 28 days and were assayed for serum electrolytes. A femoral arterial catheter was placed in each pup at 28 days for determination of mean arterial blood pressure. On days 6, 13, and 20 of lactation, the foster dams were observed for diet induced alterations in maternal behavior.

The results indicated that dietary calcium had a significant effect on pup blood pressure at weaning. Pups suckling low calcium dams had the highest blood pressures while high calcium pups developed the lowest

blood pressures. The pups displayed no dietary induced alterations in serum electrolytes at 16 days of age. However, by 28 days of age the pups suckling high calcium dams developed high serum total and ionized calcium and low phosphorus and magnesium. Low calcium pups, on the other hand, displayed low calcium and high phosphorus and magnesium.

Although the pre-pregnant females experienced nearly identical electrolyte alterations, they did not show any changes in blood pressure. The maternal behaviors were not altered by the dietary calcium manipulations.

The results indicate that perinatal dietary calcium supplementation acts to lower blood pressure in young SHRs, whereas calcium restriction acts to elevate blood pressure. Numerous factors, including altered milk electrolytes and milk hormones, as well as solid food consumption, may have been responsible for the blood pressure alterations. Although the results support some of the above factors, further research will be necessary to elucidate which, if any, are the primary mechanism of blood pressure alteration in the pups.

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OVERVIEW

The purpose of the present study was to determine the influence of dietary calcium on the development of blood pressure in neonatal spontaneously hypertensive rats. To provide a context for the study, the initial part of the introduction will briefly review the mechanics of arterial blood pressure regulation. This review is meant to supplement the following discussion, and is not intended to provide a complete description of pressure regulation. Following this will be an overview of the various forms of human hypertension. Included here will be a listing of the animal models created to better understand the human condition. Of the models discussed, the mechanisms of hypertension in the SHR will be most extensively examined. The final part of the introduction, leading to the rationale, will focus on the interaction between dietary calcium and blood pressure in the SHR.

A.1 ARTERIAL BLOOD PRESSURE CONTROL: THE BASICS

The simplest conceptual model for the vascular system is a closed loop of tubing which is filled with blood. A pump (the heart) is

attached at some point, and it functions to keep the blood continuously circulating through the loop. A constriction in the tubing at some point downstream from the pump could represent the total peripheral resistance (TPR) of the arterioles and capillary sphincters. The length of tubing running downstream from the pump to the constriction represents the arterial system, and the length running from the constriction back to the pump is the venous system. In this model, the blood pressure on the arterial side of the constriction can be increased or decreased, at a constant cardiac output (CO), by increasing or decreasing the TPR. The arterial blood pressure can also be modulated by alterations in the CO. An increase in output, at a constant TPR, will force more blood into the arterial side of the vascular system resulting in elevated arterial blood pressure. A decrease in cardiac output decreases the arterial blood pressure. In summary, the arterial blood pressure can only be altered by changes in the TPR and CO (Berne and Levy, 1981; Heller, 1981; Eckert, 1978).

A.2 SHORT TERM: INTRINSIC REGULATION

Both intrinsic (local) and extrinsic systems work together to control and modulate arterial blood pressure. Intrinsic regulation is the local response of arterioles to changing metabolic conditions. With intrinsic regulation, flow can be adjusted via local vascular resistance changes for regional fluctuations in the metabolic activity of a tissue. Fluctuating regional perfusion pressures with no change in local metabolic activity can also be met with changes in vascular resistance to insure a constant blood flow. Note that changes in arterial blood

pressure caused by intrinsic regulation are due only to changes in TPR. Intrinsic regulation is also known as autoregulation (Berne and Levy, 1981).

A.3 SHORT TERM: EXTRINSIC REGULATION

Arterial blood pressure can also be controlled extrinsically, that is, from regulation originating at sites other than local tissue. Extrinsic control of arterial blood pressure can occur through both short and long term adjustment. Many mechanisms are involved in short term regulation. Contributing elements include the outputs of the sympathetic and parasympathetic divisions of the autonomic nervous system and the prostaglandin and kallikrein-kinin systems (Berne and Levy, 1981).

The sympathetic nervous system exerts vasoconstrictive blood pressure control through neural innervation to most of the arteries in the body. In addition, an increase in sympathetic venomotor tone acts to displace blood from the venous reservoirs towards the heart which increases preload and elevates CO via the Starling mechanism. An elevated sympathetic tone also stimulates the release of epinephrine and norepinephrine from the adrenal medulla. These catecholamines, mostly through the effect of norepinephrine, stimulate a general vasoconstriction as they circulate through the vasculature. The parasympathetic division of the autonomic nervous system innervates only a small proportion of resistance vessels and thus has only a small effect on vascular resistance (Berne and Levy, 1981; Heller, 1981).

Recent evidence has demonstrated that a prostaglandin system may

operate in acute control of arterial blood pressure. Prostaglandins are small lipophilic compounds, one or more of which are normally secreted by the kidneys and other tissues. Two of these compounds, PGE₂ and PGA, act to control arterial blood pressure directly by vasodilation and indirectly by promoting excretion of salt and water. A compensatory synthesis of these prostaglandins may occur in situations where local pathologic vasoconstriction causes ischemia (Caris, 1985). The kallikrein-kinin system works in parallel with the prostaglandin system to stimulate and control widespread vasodilation (Cowley, 1985). Kallikrein is an enzyme that is found in glandular tissue throughout the body. In the blood stream, this enzyme acts to cleave kininogens to kallidin and bradykinin, both of which are vasodilators.

The extrinsic control mechanisms mentioned thus far utilize only changes of peripheral resistance. The sympathetic and parasympathetic nervous systems also modulate arterial blood pressure through direct control of the cardiac output. Increased sympathetic tone to the heart increases the heart rate. In addition, the contractility of the individual myofibrils are increased which elevates the stroke volume. These factors combine to increase the cardiac output which causes an increase in blood pressure. An elevation in vagal tone, on the other hand, initiates a slowing of the heart and has a depressive action on contractility thus causing a decrease in cardiac output and blood pressure (Berne and Levy, 1981).

A.4 LONG TERM REGULATION

Long term regulation of arterial blood pressure is controlled mainly

by the kidney. Among other functions, the kidney has the ability to filter and selectively reabsorb blood fluids, and thus can exert tight control over the blood volume. Whether blood volume is increased or decreased is determined by the levels of various circulating hormones and by the existing arterial blood pressure. A drop in blood pressure automatically causes the kidney to conserve fluids and electrolytes and this reflex acts to oppose any further fall in pressure. Elevated blood pressure, on the other hand, initiates a pressure natriuresis and diuresis resulting in a reduced blood volume and reduced pressure. Although the preceding statement is a fair summary, the actual change in arterial pressure caused by a change in blood volume is the result of a complex interaction between the kidneys, the Frank-Starling mechanism of the heart, and the autoregulatory properties of the vasculature (Berne and Levy, 1981).

The kidney also responds to a fall in blood pressure by releasing an enzyme called renin. A protein precursor circulating in the plasma, angiotensinogen, is converted to angiotensin I by the released renin. Angiotensin I is transformed into angiotensin II, the active form, by converting enzymes. Angiotensin II influences renal function by stimulating the release of aldosterone from the adrenal cortex and by a direct action on the renal tubules. Furthermore, angiotensin II has a potent vasoconstrictive effect. Angiotensin II and aldosterone act on the tubules of the kidney to speed up the reabsorption of sodium, and passively, the reabsorption of water. Thus, the end result of this feedback system is an elevation of blood pressure caused by increased vasoconstriction, and by an increase in blood volume (Eckert, 1978; Heller, 1981). The renin-angiotensin system can therefore be thought of

as both a short term (vasoconstriction) and long term (blood pressure increase) homeostatic control mechanism.

Recently, another blood volume regulatory mechanism has been proposed. This system involves communication between the brain and the kidneys via a 'natriuretic hormone'. This hormone is thought to be a 1000 M.W. protein released from the anteroventral third ventricle (AV3V) region of the hypothalamus (Bealer, 1983). Presumably, an expansion of the extracellular fluid volume, as might occur with dietary salt loading, triggers the release of this hormone. In the kidneys the natriuretic hormone acts to increase the excretion of sodium in the urine by inhibiting the Na/K ATPase located in the renal tubules. The resultant elevated sodium excretion (natriuresis) acts to counterbalance the original excess of blood volume.

Another volume regulatory hormone, the atrial natriuretic factor (ANF), has also been recently characterized (Mills, 1984). This protein, which is stored in secretory granules in the atria of the heart, is thought to be released in response to blood volume expansion. The ANF is then carried in the bloodstream to the kidneys where it inhibits the reabsorption of sodium in the distal tubules. In contrast to the natriuretic hormone, ANF does not inhibit the Na/K ATPase membrane pump. The mechanism of ANF induced natriuresis is not yet understood. In addition to the renal effects, ANF has also been shown to have a vasodilator action on vascular smooth muscle.

B.1 HYPERTENSION

Because of its varied and complex nature, hypertension is commonly

divided into secondary and essential forms. Secondary hypertension includes the forms of high blood pressure where the mechanisms are well understood (Mangery, 1982). The defects commonly seen in secondary forms include kidney disease and adrenal gland disease. For example, in certain forms of kidney disease, the renal tubules lose the ability to excrete sodium normally. This results in sodium retention which causes an increase in blood volume and blood pressure (Hall, 1980). A disease of the adrenal gland can cause an elevated aldosterone secretion which also leads to a pathologic increase in sodium retention (Weber, 1981). Pheochromocytomas are rare tumors of chromaffin tissue that excrete excessive amounts of catecholamines which cause generalized vasoconstriction and extremely high blood pressures (Meurer, 1982).

In essential hypertension, the causes of high blood pressure are unknown. Most researchers agree, however, that this disease involves a substantial genetic component that is subject to environmental influences. More than 90% of all hypertension is essential hypertension (Cowley, 1985).

B.2 EXPERIMENTAL MODELS: PHYSIOLOGICAL

Because there is so little known about the mechanisms involved in essential hypertension, there have been many animal models created to study the underlying pathologies involved in this disease. Some models have been created by altering kidney function. In Goldblatt hypertension, the renal artery of one kidney is surgically constricted

and the contralateral kidney is removed (Rosenthal, 1982). These manipulations result in increased plasma renin activity, blood volume expansion, and elevated blood pressure. 'DOCA' hypertension produces sodium retention through administration of a diet high in salt combined with infusions of a mineralocorticoid, deoxycorticosterone acetate (DOCA), which has the same actions as aldosterone (Elghozi, 1982).

Some researchers believe that the 'natriuretic hormone' is involved in the hypertension of these sodium retention models (Blaustein, 1984). It is postulated that chronically elevated levels of the natriuretic hormone will be seen in animals that express chronic sodium retention. The function of the natriuretic hormone in these animals would be to reverse the tendency towards sodium retention and to bring the system back to equilibrium. It is hypothesized, however, that the Na/K ATPase of vascular smooth muscle as well as that in the renal tubules may be chronically inhibited by elevated levels of the natriuretic hormone. This action could both depolarize the smooth muscle cell and allow for an intracellular build-up of Na, both of which lead directly to an increase in excitability and contraction of vascular smooth muscle.

Manipulations of the nervous system in animals can also produce hypertension. Such procedures include interference with vasoregulatory tracts in the brain stem, such as the nucleus tractus solitarius (Dobra, 1973), and chronic stimulation of the stellate ganglion (Rohse, 1957).

B.3 EXPERIMENTAL MODELS: GENETIC

In addition to these physiologically created models, there are a growing number of genetic models that have been bred to mimic human

essential hypertension. The New Zealand strain was developed in the mid-1950's from a colony of Wistar rats. The animals from this strain are born with elevated blood pressures compared to normotensives and they reach stable high levels (average 180 mmHG) by about six to eight weeks of age (Phelan, 1968). The Dahl salt sensitive strain was bred for a susceptibility to hypertension when administered a diet high in salt (Dahl, 1962). These rats have a genetically bred inability to excrete sodium normally. Although Dahl rats develop hypertension spontaneously, the final level of blood pressure achieved is very sensitive to salt intake. The Milan hypertensive strain, created in 1964, is similar to the Dahl strain in that the hypertension is caused primarily by impaired renal function (Bianchi, 1984). The Lyon strains bred for low, moderate, and high blood pressures, and the Sabra strains bred for a sensitivity to DOCA-salt diets, are two further examples of genetic hypertension (Ben-Ishay, 1984; Vincent, 1984).

B.4 THE SPONTANEOUSLY HYPERTENSIVE RAT

The most widely studied model of genetic hypertension is the spontaneously hypertensive rat (SHR). This strain was created in 1963 by meticulous brother to sister inbreeding of Wistar-Kyoto rats (Okamoto & Aoki, 1963). The blood pressure of SH rats, which is slightly elevated at birth, develops to abnormally high levels by about five weeks of age (Gray, 1984). The systolic blood pressure of these rats in adulthood is in the range of 180-210 mm HG.

Because of the many pathophysiological similarities between the SHR and human essential hypertension, this strain is perhaps the best

available animal model for essential hypertension (Trippodo, 1981). For instance, in SHR and essential hypertension alike, blood pressure is slightly elevated in the young (Gray, 1984) and it increases with age to finally terminate with heart failure, arteriolosclerosis, and renal insufficiency (Trippodo, 1981). Other similarities between the SHR and human hypertension include increased total peripheral resistance and normal cardiac output (Simpson, 1976), increased vascular reactivity to environmental stressors (Trippodo, 1981), similar alterations in vascular smooth muscle membrane and a shortened life span (Trippodo, 1981). Furthermore, in both types of hypertension, a strong underlying genetic component has been demonstrated (Trippodo, 1981).

Intensive studies over the past twenty years have demonstrated many organ and tissue differences between SHRs and normotensive controls. However, the process of forming theories about what actually causes hypertension based on these differences has been complicated by two inherent problems. One problem recognizes that the development of hypertension in an organism causes compensatory changes in nearly all of the organ systems. Therefore, in most cases it is difficult to tell whether an abnormality is a primary cause of hypertension or a compensatory change. The other problem emerges from the fact that SHRs are an inbred strain. The process of selection and inbreeding for hypertension necessarily selects for other specific traits that have no relation to hypertension. These 'extraneous' traits can easily be confused with causative factors in hypertension.

Most researchers agree that the elevated blood pressure of SHRs is due to an increase in total peripheral resistance (Simpson, 1976; Wright, 1981). The blood volume and CO in SHRs are normal and even

sometimes reduced as compared to normotensive controls (Ferrone, 1979; Sen, 1972). Cardiac hypertrophy does develop in later life, but this is thought to be a compensatory response to the increase in peripheral resistance (Simpson, 1976).

Alterations in one or many of the cardiovascular regulatory systems could be responsible for the increased vascular resistance seen in SHR. For example, modifications in the renin-angiotension system and in the prostaglandin and kallikrein systems as well as the sympathetic nervous system could all lead to an increase in peripheral resistance. Increased resistance could also be caused by a fundamental change in the membrane or in the contractile apparatus of the vascular smooth muscle cell.

B.5 SHR: ALTERED SYMPATHETIC FUNCTION

The results from an early set of studies suggested that SHRs might have altered sympathetic function (Hallback, 1974). These studies reported that SHRs respond to alerting stimuli with larger increases in heart rate, stroke volume and arterial pressure than normotensive controls. Since then, it has been demonstrated that the development of high blood pressure in SHRs can be reduced by chemical (Provost, 1978) or immunosympathectomy (Cutilleta, 1977). Reductions in blood pressure can also be achieved by induced depletion of central catecholamines (Erinoff, 1975). Other lines of evidence including direct recordings have revealed increased activity in both pre and postganglionic fibers of the sympathetic nervous system in SHRs (Schramm, 1979). In addition, SHRs respond with an enhanced release of catecholamines when challenged

with behavioral stressors (Kvetnansky, 1979). An interesting recent study revealed that SHRs release more norepinephrine from their postganglionic sympathetic nerve terminals for a given electrical stimulus (Lokhandwala, 1983). The authors speculated that the elevated levels of circulating epinephrine commonly seen in SHRs might stimulate postganglionic presynaptic beta 2 adrenergic receptors resulting in enhanced norepinephrine release from the presynaptic terminals.

Folkow (1982) has pointed out that elevated transient pressor responses do not in themselves cause sustained high blood pressure. The chronic hypertension seen in the SHR may, however, be explained as the result of 'structural autoregulation' of precapillary vessels to frequent inductions of elevated pressor responses. Structural autoregulation, an observed phenomenon, is an adaptive increase in the wall/lumen ratio of resistance vessels as a local response to sustained increases of average transmural pressure.

In summary, there is little doubt that increased sympathetic activity is partly responsible for the increased peripheral resistance and elevated blood pressure seen in the SHR. It is not yet clear, however, whether the increased sympathetic activity arises centrally, peripherally or both.

B.6 SHR: ALTERED VASCULAR SMOOTH MUSCLE MEMBRANE

Another clue in the causation of enhanced peripheral resistance in SHRs came from studies that showed hyperresponsiveness of SHR vascular smooth muscle to vasoconstrictor stimuli. When isolated strips of SHR vascular smooth muscle are challenged with various vasoconstrictor

agents such as norepinephrine, angiotension II, and vasopressin, the preparations respond with more contractile force than strips isolated from normotensive rats (Bohlen, 1979; Collis, 1977). This hyperfunction suggests that there may be one or many abnormalities in the smooth muscle membrane of SH rats.

A brief review of the mechanics of smooth muscle contraction will aid in the understanding of the membrane abnormalities seen in the SHR. The steps in contraction of smooth muscle are as follows: (1) calcium rushes into the cell through voltage and receptor gated channels (2) the inflowing calcium triggers a further release of calcium sequestered by cell organelles, such as the sarcoplasmic reticulum (SR) (3) increased levels of intracellular calcium complex with and activate calmodulin which in turn activates myosin light chain kinase (4) this enzyme complex activates one of the light chains of myosin which triggers the cross linking of the contractile proteins ultimately leading to smooth muscle contraction (Bohr, 1984). These mechanisms are related such that, over a specific range of concentration, a small change in intracellular ionized calcium causes a large change in the state of contraction of vascular smooth muscle (Blaustein, 1977). The calcium levels inside the cell are maintained at low levels between contractions by a membrane bound calcium ATPase, a membrane bound Na/Ca antiport system, and by sequestration into the SR and mitochondria. The calcium ATPase actively pumps calcium out of the cell against its concentration gradient. This pumping requires energy in the form of ATP available for dephosphorylation. Calcium is also pumped out of the cell by the Na/Ca antiport system, and here the energy is provided by Na flowing inward, along its concentration gradient. Available intracellular

calcium is also removed by active pumping into the SR and mitochondria. It should be noted that the ubiquitous Na/K ATPase is also located in smooth muscle membrane where it serves two cell stabilization functions. Because this pump is electrogenic, that is, it extrudes three sodium ions for every two potassium ions pumped in, it acts to hyperpolarize the cell. This hyperpolarization acts to decrease the probability that the voltage gated calcium channels will be open, and therefore it slows the influx of extracellular calcium. A second stabilization function derives from the fact that the Na/K ATPase helps to maintain the large Na concentration gradient across the cell membrane. The Na/K ATPase thus indirectly helps to extrude calcium from the cell because the Na/Ca antiport system depends on the Na concentration gradient (Rasmussen, 1984).

Extensive research has revealed many abnormalities in membranes isolated from SHR tissue. In terms of passive fluxes, many researchers have found an increase in the permeability to ionic calcium in SHR membranes (Herder, 1983; Hinke, 1966; Hollaway & Bohr, 1973; Wright, 1981). SHR membranes have also been found to be 'leaky' to sodium and other monovalent ions (Haddy, 1983).

Active transport mechanisms are also altered in SHRs. Kwan (1980) has demonstrated that the ATP dependent extrusion of calcium is reduced in SHR smooth muscle membranes. In addition, it has been demonstrated that the sequestration of calcium into the sarcoplasmic reticulum is reduced in SHR smooth muscle (Wright, 1981). The literature on Na/K ATPase activity is conflicting. Some experimenters have found an increase in Na/K ATPase in SHR vascular smooth muscle (Gothberg, 1980; Smits, 1982), while others have found a decrease in activity in

erythrocytes (Sowers, 1983) and in cardiac tissue (Godfrained, 1980; Lee, 1983).

Any or all of these abnormalities could contribute to the increased vascular resistance seen in the SHR. An increased passive flux of calcium into a vascular smooth muscle cell could directly lead to increased constrictor tone. The increased inward sodium flux could lead to increased tone indirectly via two routes: (1) the inward flow of positive sodium ions would tend to destabilize the cell membrane, and (2) the consequent reduction in the Na concentration gradient across the cell membrane would slow the function of the Na/Ca antiport leading to a reduction of calcium extrusion. If decreased Na/K activity does indeed exist in SHR smooth muscle membrane, then this abnormality could lead to membrane depolarization and muscle contraction through a reduction in its electrogenic activity and a reduction in the sodium concentration gradient across the membrane.

Studies looking at the cellular handling of calcium have suggested new theories describing the development of hypertension in the SHR. Elevated extracellular ionized calcium is known to have a 'membrane stabilizing effect' (Webb, 1978). Empirically, these studies have shown that elevated calcium decreases the response of helical strips of rat tail artery to constrictor agents, whereas lowered calcium has a destabilizing effect. Further research has revealed that calcium binds to specific loci in the extracellular surface of the membrane and this binding is believed to decrease the membrane permeability to monovalent ions (Jones, 1974). Decreased calcium binding to the membrane, on the other hand, is associated with increased membrane permeability to monovalent ions. In SHRs, the calcium binding to vascular smooth muscle

fragments is reduced (Bohr, 1984), and it has been shown that SHRs require higher levels of calcium to attain the same level of smooth muscle relaxation as in normotensive strains (Hansen, 1975). In addition, SHR membranes are more permeable to monovalent ions. Given the above hypothesis, it is possible that an abnormality in the membrane binding of calcium could at least partially account for the increased contractility and reactivity of SHR smooth muscle.

A related finding by McCarron (1981) was that SHRs consistently exhibit reduced blood plasma levels of ionized calcium and normal total plasma calcium levels as compared to WKY controls, and that a reduction in dietary calcium caused an elevation in blood pressure in SHRs (McCarron, 1982b). This was taken as further evidence that SHRs have some basic abnormalities in calcium binding. A further finding was that dietary supplementation of calcium in SHRs may compensate for their genetic defect in calcium binding by raising their plasma ionized levels to normal values. This intervention also retards and/or prevents the attainment of the normally expected high blood pressures found in SHRs (McCarron, 1981). These results are in nice agreement with the theory that higher levels of calcium cause vascular smooth muscle membrane stabilization which decreases the contractility of these cells to ultimately result in lowered of blood pressure. Studies with WKY rats, a genetically related normotensive strain, have also revealed an inverse relationship between dietary calcium and blood pressure (McCarron, 1982a).

Further research has revealed that calcium supplementation in young SHRs may be more effective in lowering blood pressures than similar manipulations at later ages. McCarron has found that calcium loading

in the diet has a much greater blood pressure lowering effect in the SHR if the diet is begun at four weeks rather than at ten weeks of age (McCarron, 1982b). These findings are consistent with other studies that have demonstrated the importance of the 'prehypertensive' stage in spontaneously hypertensive animals. Interest in this area was stimulated by findings showing many abnormalities in neonatal SHRs including significantly raised blood pressures (Gray, 1984), and increased adrenal medullary, mineralocorticoid, and renin-angiotension function (Gray, 1984; Simpson, 1976).

Taken together, these studies suggest that there is not an early "prehypertensive" period in the SHR as postulated by Okamoto and Aoki (1963), but rather the mechanisms that cause hypertension seem to be in action even at birth. Supporting this hypothesis is a study that examined the early influence of sodium in SHRs (Karr-Dullien & Bloomquist, 1979). Here it was shown that SHR pups born from dams on a high sodium diet developed abnormally high blood pressure when they were continued on a high sodium diet post weaning (Karr-Dullien & Bloomquist, 1979).

In the context of these findings, a recent experiment demonstrating a potential hypertensive factor transferred from lactating SHR dams to their fostered pups becomes increasingly interesting (McMurtry, 1981). In this study, McMurtry took SHR and normotensive litters and exchanged them at birth so that the normotensive pups were suckled on SHR dams and vice versa. By using the proper cross fostering control groups, McMurtry was able to separate out both prenatal and postnatal variables along with the effect of fostering itself. The significant result of this study was that genetically normotensive animals become hypertensive

if they were nursed by a hypertensive mother.

The authors speculated that either a hypertensinogenic factor was transmitted through the SHR milk, or some behavioral abnormality of the SHR dams induced higher blood pressure in the normotensive animals during suckling. Past evidence, in fact, support both of these contentions. In terms of behavioral abnormalities, SHRs are generally more active, and exhibit more aggressive behavior when isolated than their normotensive counterparts (LeDoix, 1982; Shimomoto, 1971). Evidence for altered humoral factors in blood plasma of SHRs that might thus be altered in the milk are more difficult to characterize. A procedure called parabiosing, in which the blood from an animal is continuously transferred to another, demonstrated that WKYs that received blood from SHRs in a five month period developed abnormally high blood pressures (Greenberg, 1981). Because the same result can be achieved by parabiosing Goldblatt and DOCA animals with normotensives, it has been suggested that the SHR as well as these fluid expanded models may exhibit elevated levels of the natriuretic hormone. In support of this hypothesis, studies have demonstrated an exaggerated natriuresis and diuresis in the SHR upon salt loading (DiBona, 1978; Francisco, 1981). Others have pointed out, however, that elevated levels of the natriuretic hormone are only found in volume expanded low-renin models of hypertension, and the SHR exhibits neither of these characteristics (Bealer, 1983). Furthermore, the hypertension displayed by the SHR is not prevented by a lesion in the AV3V region of the brain, a site known to be involved in the release of the natriuretic hormone (Haddy, 1983; Harder, 1983).

Other possible hypertensinogenic factors in SHR plasma include

elevated levels of parathyroid hormone as well as two recently isolated plasma proteins from SHRs that were not found in WKY plasma. (Cloix, 1983; McCarron, 1981; Trippado, 1981).

Although all of these factors could play a role in the suckling induced hypertension reported by McMurtry, there is another possibility found in the disrupted calcium metabolism of SHRs. The hypothesis is simply that SHRs exhibit an altered calcium metabolism which could be reflected as reduced calcium transmitted in the milk. As a diet low in calcium has been found to increase blood pressure in SHRs and in normotensive strains, so could a diet of milk low in calcium have a like effect.

During the reproductive cycle in the rat, a substantial amount of calcium is required for fetal development and neonatal growth. This calcium must be supplied by the female rat through the placenta during pregnancy and through the milk during the suckling period. Several mechanisms come into play in the female to assure that the neonate will receive enough calcium. These mechanisms include an increased intestinal absorption of calcium via increased food consumption, stimulation of active calcium transport in the duodenum, increased permeability of the jejunum to calcium, and selection of an adequate calcium diet (Brommage, 1985). During late pregnancy and lactation, the dietary sources of calcium are not sufficient for the neonatal demand and the remaining calcium must come from the maternal skeleton (Brommage, 1985; Halloran, 1981). The tremendous strain that this period puts on the maternal calcium compensatory mechanisms is evidenced by the fact that nearly one half of the maternal skeletal mineral is lost in lactating rats put on low calcium diets (Ellinger, 1982).

Furthermore, lactating rats sometimes display a severe hypocalcemia (Toverud, 1976).

Spontaneously hypertensive rats clearly have some defects in their adult calcium compensatory mechanisms. An example is given by the fact that older SHRs display a chronic exaggerated loss of calcium in the urine in the face of abnormally high parathyroid hormone levels (McCarron, 1981). It will be recalled that one of the functions of the parathyroid hormone is to decrease urinary excretion of calcium. In addition, in response to a low calcium diet, urinary calcium excretion fails to diminish in the SHR as it does in the WKY (Shneidman, 1984). As discussed before, SHRs also exhibit many calcium regulatory abnormalities at the cellular level. In view of these abnormalities, it is not unlikely that SHRs might exhibit calcium regulatory abnormalities during pregnancy and lactation resulting in reduced available calcium for secretion into the milk. In light of McCarron's study which showed that calcium supplementation has its greatest effect when given to the young animal, an altered milk calcium availability could have profound effects on the development of hypertension in the developing SHR pup. To test this hypothesis, an experiment was designed to alter the calcium diets of lactating SHR dams. It was postulated that the altered dietary calcium may be reflected in the milk and expressed as a hypertensinogenic factor for the pups.

C. PRELIMINARY RESULTS

In a pilot cross fostering study, 12 SHR females were fed a diet high in calcium (2% by weight) and 11 were fed a low calcium diet (0.1%). After approximately 14 weeks on the diets, indirect tail cuff blood pressure measurements were made, and blood samples were taken for analysis of serum ionized and total calcium. At two days following this procedure, the females were mated with 11 male SH rats. The mating procedure consisted of housing one male to every two females in regular gang cages. All males were placed with the females at the same time and they were removed after 6 days of exposure. The births occurred over a 5 day period and all litters were fostered as soon as possible after parturition. Most litters were fostered within a day and many were fostered at birth. The fostering procedure consisted of splitting each litter in two, giving half of the litter to a foster mother of the opposite diet condition and giving the other half to a foster mother of the same diet condition. This design, known as "cross fostering", was used to separate the gestational and neonatal influences of dietary calcium. If, for instance, gestational influences dominated, then the effect of the gestational calcium diet would be seen at weaning, regardless of the assigned lactational condition. If, however, a gestational-lactational interaction occurred, then the pup groups that were "crossed" from a given gestational condition to a different lactational condition would demonstrate a modulation of the gestational influence. Pups are always "fostered" to nonbiological mothers in this

type of design so that fostering itself can be ruled out as a variable.

At birth, each pup was sexed, weighed, and given a tattoo to demarcate its maternal origin. All pups were weighed again at 6 days following birth and on every 6th day thereafter. At 7 days postpartum an attempt was made to obtain milk samples from the dams in order to analyze the milk for total and ionized calcium content. Each dam was separated from her litter for 4 hours following which an intravenous injection of oxytocin was given to stimulate milk let-down. The dams were then milked, under ether anesthesia, using a short length of polyethylene tubing connected to a vacuum pump. A small glass test tube was connected in series between the pump and tubing for collection of the milk.

At weaning (27-29 days postpartum) direct blood pressure measurements were taken on each pup using indwelling arterial catheters. The catheters were made of a 3 cm piece of P.E. 10 tubing welded to a 10 cm piece of P.E. 50 tubing. The P.E. 10 length of tubing was placed in the left femoral artery of each pup, and the P.E. 50 length was tunneled subcutaneously to exit out of a dorsal neck incision. At 20 minutes following surgery, direct blood pressure measurements were taken on conscious animals for determination of mean arterial blood pressure. Immediately following pressure measurements, the dead space fluid was bled out of the catheter of each pup and 1.5 mls of blood were then collected for determination of serum ionized and total calcium.

Blood samples taken from the prepregnant dams revealed a significantly higher serum ionized and total calcium content in the high calcium diet group as compared to the low group (ionized Ca, $t(21)=2.2$, $p<.05$; total

Ca, $t(21)=2.3$, $p<.05$). Blood pressures, however, did not differ significantly between the two groups. The pregnancies and the fostering procedure went well. A 70% pregnancy rate was obtained with an average of 7.1 pups born to each litter. The milk sampling was unsuccessful because the quantities of milk collected were too small for analysis of calcium levels.

At 28 days of age, a 2 by 2 factorial analysis of variance (ANOVA) of blood pressure revealed that pups suckled to high calcium dams ($N=12$) had significantly lower blood pressures ($M=104.6$ mmHG) than pups suckled to low calcium dams ($N=21$, $M=129.2$ mmHG), $F(1, 29)=17.4$, $p<.01$. There was no effect for biological origin nor was there a biological origin by fostering interaction. An ANOVA of blood calcium content demonstrated that pups suckled to high calcium dams had higher serum ionized ($M=1.13$ mM/l) and total ($M=10.2$ mg/dl) calcium than pups suckled to low calcium dams ($M=0.92$ mM/l ionized, $M=8.8$ mg/dl total), $F(1, 29)=28.8$, $p<.01$ ionized; $F(1, 29)=13.4$, $p<.01$ total. Biological origin and interaction effects were not significant.

The results suggest that the calcium diets may have a differential effect on the maternal milk content such that pups receiving milk from high calcium diet mothers have lower blood pressures and higher total and ionized blood calcium values than pups suckled to low calcium mothers. Any in-utero influences that maternal dietary calcium may have had were not detectable at 28 days of age.

Maternal cannibalism and neglect caused an unusually high death rate (70%) among the pups. An obvious cause of these maternal behavior deficits was the harassment produced by the sequential weighing and milking procedures. Furthermore, a study by Denenberg demonstrated that

pup survivablility can be greatly increased if pups are allowed to stay with their biological mothers for 12 hours before fostering (Denenberg, 1963). A fostering study that omits the weighing and milking procedures and allows 12 hours of exposure with natural mothers would probably obtain better survivability among the pups.

In the absence of a control diet group, it is difficult to make any conclusions about the blood pressure raising or lowering effects of the high and low calcium diets. The inclusion of a normal calcium diet group would provide a control condition from which to compare the other two diets.

D. RATIONALE

The results from the preliminary experiment suggest that early postnatal manipulations of dietary calcium have a substantial influence on the cardiovascular physiology of developing SHR pups. Therefore, the purpose of the present study was to replicate and improve the postnatal portion of the preliminary experiment. Based on the preliminary results, it was expected that pups suckled to high calcium dams should exhibit higher blood calcium values and lower blood pressures than pups suckled to low calcium dams. Similar fostering studies have demonstrated that high maternal salt diets and high maternal cholesterol diets can partly be transferred to developing pups via suckling (Karr-Dullien, 1979; Naseem, 1980).

With only a postnatal manipulation in the present experiment, room was available for improvements and expansions on the previous design. For example, whole litters were fostered from medium calcium dams to dams on the low, medium and high diet conditions. Two pups were then randomly chosen from each fostered litter at 28 days postpartum for determination of arterial blood pressure. This design had the advantage of being simpler than the previous experiment, and it ensured that the litters were equally represented in the three experimental groups. In addition, the medium calcium group, which was added from the previous study, was intended to provide a control condition from which to compare the blood pressure raising and lowering effects of the high and low calcium diets.

A further improvement was a maternal behavioral analysis. Because

calcium induced alterations in behavior were not expected, maternal behavior was not anticipated to be a factor influencing pup blood pressure in the present experiment. Finally, blood samples were taken from suckling pups at 16 days of age, which is the earliest time that rat pups begin ingesting small amounts of solid diet (Redman, 1976). If the pup electrolytes were influenced by the suckling conditions at 16 days, then it can be assumed that the maternal calcium diets had an impact on the milk nutritional content.

E. METHOD

E.1 Subjects

Sixty-six female and 25 male six week old spontaneously hypertensive rats were obtained from Charles River Breeding Laboratories. Upon arrival, the female SHRs were divided into three diet groups: one group receiving a high (2.0%) calcium diet (% dry weight), another group receiving a medium (1.0%) calcium diet, and a third group receiving a low (0.1%) calcium diet. Thirty-six females were assigned to the normal diet, and 15 females each were assigned to the high and low diets. The female subjects remained on their respective diets for the duration of the experiment. The male SHRs were fed regular rat chow and both sexes received water ad libitum. All animals were maintained on a 12:12 light/dark cycle.

E.2 Determination of pre-pregnancy BP and plasma values

After six weeks on the special calcium diets and at two days prior to breeding, blood samples were taken from the females for determination of serum electrolytes. Each subject was put under light ether anesthesia following which 3.0 to 3.5 mls of blood was removed via a subclavian venapuncture. Isotonic saline replacement, 3 to 3.5 mls, was given subcutaneously at the abdomen after phlebotomy. Blood samples were placed separately in labeled glass test tubes and were set aside for 60

minutes to allow for coagulation. Samples were then placed in a centrifuge (Philips-Drucker, Astoria, Oregon) and spun at 20-25 thousand rpm for 15 minutes. The supernatant serum was drawn off with a disposable pipette and the red blood cells were discarded. The serum was assayed for ionized Ca using a calcium specific electrode (Radiometer America, Westlake, OH). Total calcium, magnesium, and phosphorus were determined by spectrophotometry (COBAS-BIO centrifical analyzer, Roche Analytical Inst., Nutly, NJ). Sodium and potassium were determined by flame photometry (Instrumentation Laboratories, Tustin, CA).

On the following day, indirect measurements of blood pressure were taken on all female subjects. Each rat was restrained in a bootstrap rat holder (IITC Inc., Landing, NJ) and placed in a temperature controlled model 300 test chamber (IITC Inc.). The ambient temperature in the chamber was maintained at 27 C, and the rats were sequestered inside for 20 minutes before the first pressure measurement. A photoelectric sensor (IITC Inc.), consisting of a miniature focused light and a photoresistive cell mounted in a 50mm long inflatable rubber cuff, was placed on the base of the tail. Pulse signals from the photocell were fed into an amplifier (model 59, IITC, Inc.), for regulation of gain, offset, and intensity of the light source. A programmed electrosphygmomanometer (model 20 IITC Inc.) was connected to the the rubber cuff to keep inflation-deflation rates constant and register pressure changes with a transducer. Signals from the photocell amplifier and cuff pressure transducer were recorded continuously on separate channels of an ink writing recorder (IITC Inc.). This method detected the first pulses that traveled across the caudal artery as the

cuff lowered in pressure. The first pulse detected corresponds to the systolic blood pressure. Four clear recordings were taken and averaged together to give one systolic blood pressure for each rat. The female SH rats were number coded to ensure that the blood pressure scoring was done blind.

E.3 Breeding

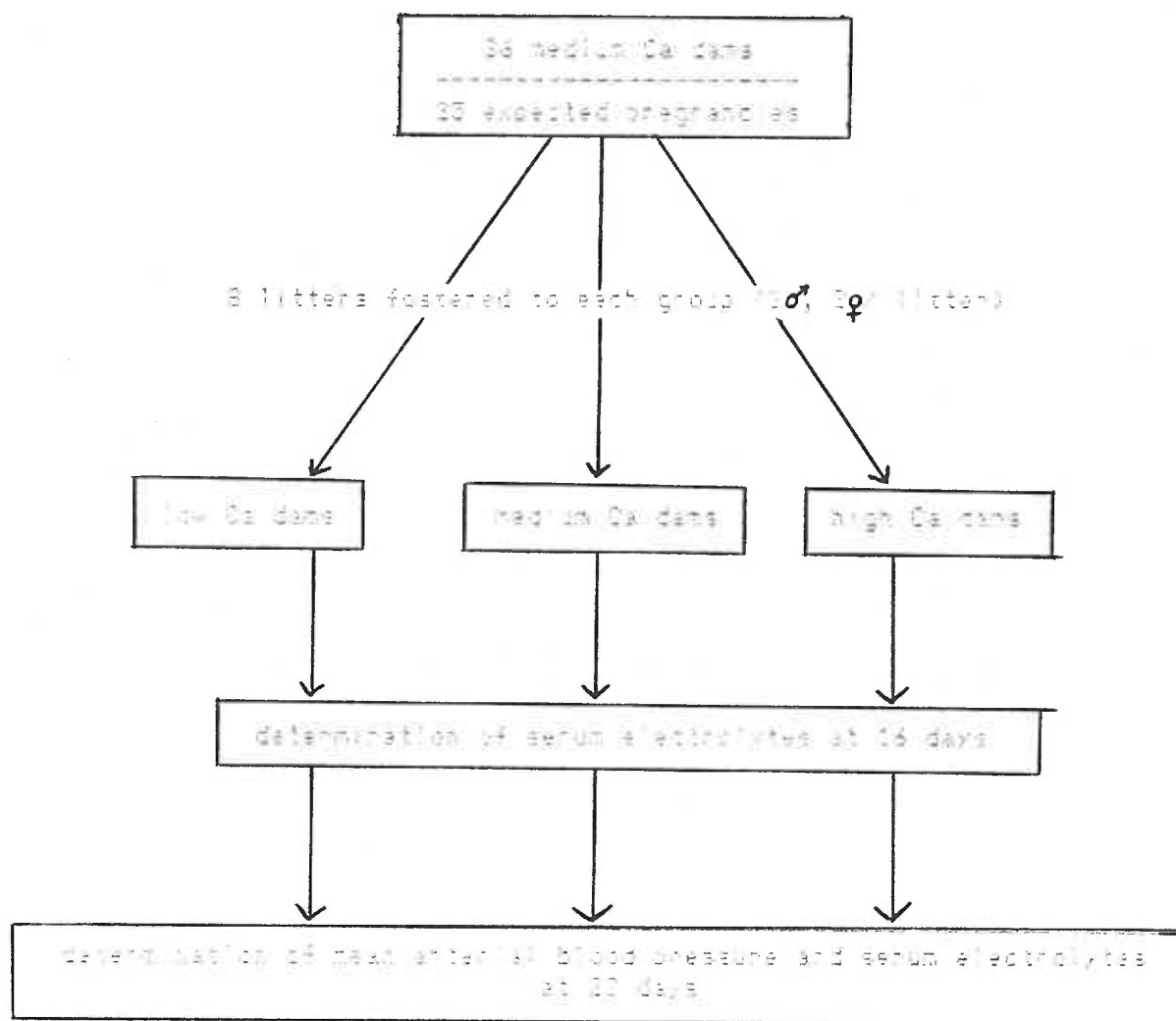
The female SHRs were housed two to a cage in standard gang cages located in a colony room with a 12 hr light-dark cycle. In order to obtain synchronous parturition, all of the males were placed in the gang cages at the same time, giving a ratio of one male rat for every two females. At ten days before the expected parturition, the females were housed individually in shoe box cages. Wood shavings and paper nesting material were provided to each mother. Food and water were available ad libitum.

E.4 Cross Fostering

At days 20 and 21 of gestation the females were watched closely for signs of impending parturition. As each litter was born, the time and date of birth were recorded. At 12 to 24 hours following birth, each litter was removed from its biological mother for cross fostering.

The fostering procedure, which is presented in Figure 1, consisted of separating litters from dams on the normal calcium condition and fostering them to dams on the low, normal, and high calcium conditions. A subset of 8 dams from the normal calcium condition were randomly

Figure 1. A schematic diagram of the fostering procedure.



chosen as the normal group to receive foster pups.

Individual fostering procedures consisted of separating normal calcium dams from their litters. In order to prevent hypothermia during this procedure, the pups were temporarily placed in a heat mantle set at 36 C. Each litter was culled to 6 pups, keeping the number of males and females as equal as possible. The culled litters were then assigned in their entirety to foster dams of either the high, normal, or low conditions. The foster dams just had their natural litters removed from them.

E.5 Maternal Behavioral Analysis

Because the different calcium diets may have an effect on the maternal behavior of the SHR dams which could influence the blood pressure and other parameters measured in the pups, an observational analysis was made on maternal behavior. Observations were made on all of the dams between hours 1-2 pm and 11-12 pm on days 6, 13, and 20 postpartum. Each observation period began by scoring the behavior of a particular dam at the beginning of a 5 second epoch. At the onset of the next epoch, the observer had recorded the previous behavior and moved in position for the next observation. This procedure was repeated until each dam had been observed 30 times. Behaviors observed and recorded include: nursing, licking-sniffing of pups, contact with pups but no nursing or licking-sniffing, and zero contact with pups. Each behavior was scored once if it occurred at the beginning of an epoch. Nursing behavior and licking-sniffing could occur together, however, simple contact and zero contact were exclusive of other behaviors.

The observations during hours 1-2 pm were made under normal white illumination (120 Watt fluorescent lights) while observations made during hours 11-12 pm were made under red illumination (a 120 Watt bulb with a red filter). The observations made during the red illumination period were in "darkness" because red light is not visible to SHR_s and other albino rats. Each dam and litter were coded and their position was shuffled to ensure that the scorer stayed unaware of the diet condition observed.

E.6 Determination of serum electrolytes at 16 days

Single male pups were randomly chosen from each litter at 16 days of age and blood samples were drawn for determination of serum electrolytes. Each pup was anesthetized with ether and a ventral midline incision was made to expose the heart. Using a 1 cc syringe and a 22 ga needle, a 1.5 to 2.0 ml blood sample was drawn directly from the heart. Following this procedure, the animals were immediately euthanized with ether. To ensure that a maximal amount of plasma was collected, the whole blood was placed in microhematocrit capillary tubes and was spun for 2 minutes in a capillary centrifuge (International Equipment Co., Needham Heights, Mass). After centrifugation, the tubes were broken and the supernatant plasma was blown into sample vials for electrolyte assay.

E.7 Determination of blood pressure at 4 weeks

Because of the difficulty in taking indirect blood pressure recordings

in very young rats, direct recordings were made using indwelling catheters. The catheters also served as a means of obtaining blood samples from the pups for determination of serum electrolytes at 4 weeks of age.

E.7.a Anesthesia. Halothane gas in oxygen was used as the general anesthetic. The oxygen flow was held constant throughout the surgery at 0.5 l/min. by an Airco oxygen flowmeter. The rate of halothane flow was controlled by a calibrated vaporizer (Ohio Medical Products).

The rat was placed in a Nalgene bottle and put down with a loading dose of halothane gas (4-5 % concentration in oxygen). Anesthesia was maintained with a 1-2 % concentration of halothane delivered to the rat through a nose cone.

E.7.b Catheter construction. The femoral artery catheter was constructed by first cutting a 20 cm length of P.E. 50 (polyethylene) tubing (0.58 mm I.D.; 0.96 mm O.D.). The end of the catheter to be inserted into the artery was heated over the flame of an alcohol burner. It was then stretched to approximately one half the diameter of P.E. 10 tubing (0.28 mm I.D.; 0.61 mm O.D.), and finally cut at the most narrow point by a scalpel. The P.E. 50 end was fitted onto a needle tipped syringe and the catheter was then ready for use.

E.7.c Surgical Procedure. The rat was shaved in a 2 cm area along the ventral surface of the left hind leg up to the ventral midline. A 1.5-2.0 cm incision was made in the shaved area superficial and parallel to the femoral artery using fine scissors. Two fine tipped curved tissue forceps were used to separate fascia and expose the femoral artery as it passed medially to join the terminal aorta. The points of the forceps were placed under the artery in order to pull two strands of

000 silk suture, 10 cm long, under the artery to use as ligatures. The ligatures were placed at either end of the exposed length of artery. The proximally placed ligature was tied in a loose single knot loop around the artery and the distally placed ligature was tied in a tight knot loop. A single drop of Papaverine (30 mg/ml) was applied to the surface of the artery to cause a relaxation of the smooth muscle in the arterial wall. A pair of curved hemostatic forceps was then attached to the ligatures and the forceps were situated to create tension on the ligatures which slightly stretched the artery and acted to cut off the flow of blood.

The beveled end of a 25 ga hypodermic needle was bent at a 90 angle to the stem of the needle. The needle was inserted into the artery so that the beveled end was parallel to the artery and the tip faced medially, opposing the flow of blood. The narrow end of the catheter (as described previously) was slid underneath the needle tip and the catheter was advanced inside the artery medially toward the terminal aorta. The needle tip was removed and the tension on the proximal ligature was released so that the catheter was able to advance into the artery for about 3 cm.

The loose single knot in the proximal ligature was tightened around the artery and the catheter so as to hold the catheter in place. The loose threads of the distal ligature were cut off and discarded. Blood flow was checked by drawing blood into the catheter and flushing saline back into the artery. To prevent clotting, a solution of one part saline and one part heparin (1000 units / ml) was flushed into the dead space of the catheter. A 2.0 cm piece of # 10 stainless steel music wire was inserted into the P.E. 50 end of the catheter while the

catheter was not in use.

The rat was then turned over and a 2 cm area on the back of the neck was shaved. A 1 cm long incision was made in the shaven area with fine scissors. A 15.5 cm long piece of 13 ga stainless steel hypodermic tubing was used to tunnel subcutaneously from the neck incision to the leg incision. The catheter was inserted into the tubing at the leg end, and the tubing was removed leaving the catheter extending out of the neck wound. The neck and leg incisions were sutured with 000 silk. At the end of the surgery, the halothane gas was turned off and the rat was given oxygen for 5 minutes.

E.7.d Apparatus. Arterial blood pressure was recorded by connecting the femoral artery catheter to a Statham p-23-Db pressure transducer. For recording purposes, the transducer was placed at the heart level of each animal. Signals from the transducer were fed to a Grass low level D.C. preamplifier which were in turn connected with a Grass chart recorder. An Interactive Structures, Inc. analog to digital device (Model al13) interfaced to an Apple II computer was used to convert the analog blood pressure signal to a digital representation. A mercury manometer was used to calibrate pressure for both the chart recorder and the computer. The device sampled blood pressure every 10 msec throughout each measuring period. The pressure values were converted to mean blood pressure in mm Hg and were stored on disk.

E.7.e Procedure. At 28 days postpartum one male pup and one female pup from each litter were implanted with a catheter. All surgeries and pressure determinations were performed between 9 am and 1 pm. However, on days when multiple catheterizations were performed, the order of surgeries were randomized for diet condition. This procedure was an

attempt to rule out the time of day as a variable in the determination of arterial blood pressure. At exactly 2 hours following the surgery, each pup was placed in a small animal restrainer and the catheter was connected to the Statham pressure transducer with a short length of P.E. 50 tubing. Following this procedure, the animals were allowed to habituate to the restrainer for 15 minutes before the first blood pressure samples were taken. Mean arterial blood pressure was measured during the first five seconds of each minute for fifteen consecutive minutes. The resulting fifteen data points were averaged together to give a single blood pressure score for each animal. Following the blood pressure evaluation, 1.5 ml of blood was bled from the catheter, and these samples were used for determination of serum electrolytes (as described previously). Because young rats are unable to develop adequate collateral hindlimb circulation following catheterization surgery, the pups were immediately euthanized with ether following each blood drawing.

F. RESULTS

Blood chemistry data from samples taken prior to pregnancy are presented in Figures 2 and 3. One way ANOVAs were utilized to detect overall diet effects and subsequent contrasts were made using Newman-Keuls tests.

As expected, dams in the low calcium condition had the lowest serum ionized calcium values while dams on the high diet had the highest values ($F_{2,63} = 45.41, p < .01$). Subsequent analysis of the data showed that all three diet conditions were significantly different from one another ($p < .01$). Although total calcium followed the same response pattern, the effect was not statistically significant ($F_{2,63} = 2.47, p = .09$).

Magnesium was inversely related to the level of dietary calcium ($F_{2,63} = 51.46, p < .01$). That is, the high calcium diet resulted in the lowest levels of magnesium whereas the low diet resulted in the highest levels. Separate post hoc analyses revealed that, as with ionized calcium, all three conditions were significantly different from one another ($p < .01$).

Serum phosphorus responded to dietary calcium in the same manner as did magnesium ($F_{2,63} = 22.96, p < .01$). The post hoc analysis for phosphorus showed that all three diet conditions were significantly different from one another with the lowest phosphorus levels found in the high diet dams and the highest levels found in the low diet dams ($p < .01$).

Figure 2. The effect of diet condition on mean (+S.D.) serum ionized calcium (A), total calcium (B), magnesium (C), and phosphorus (D) in pre-pregnant SHR dams.

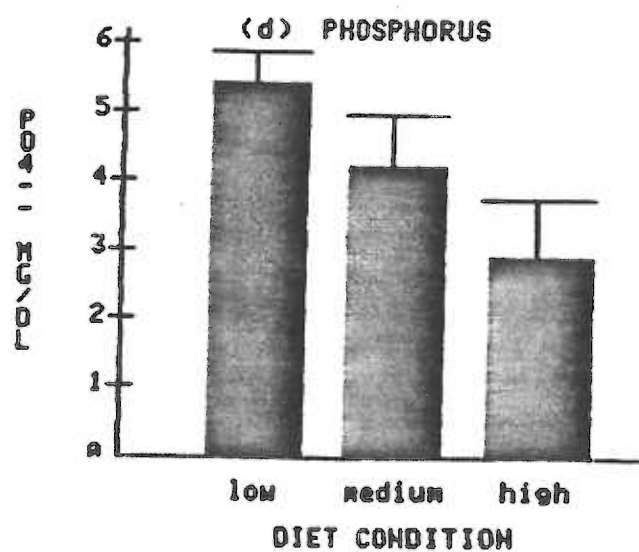
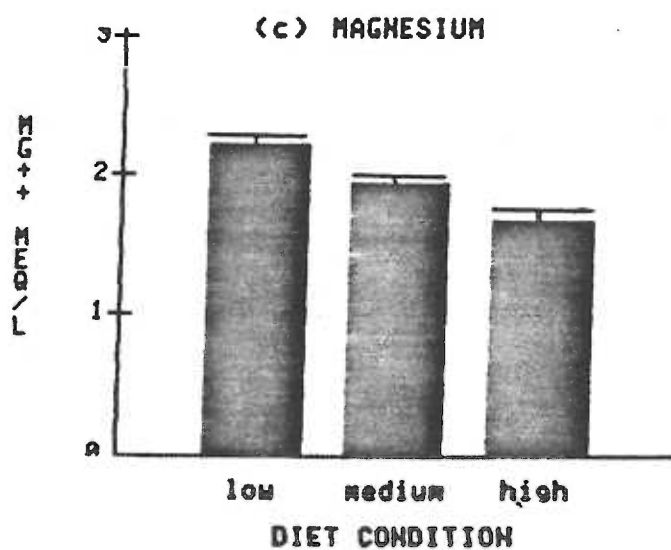
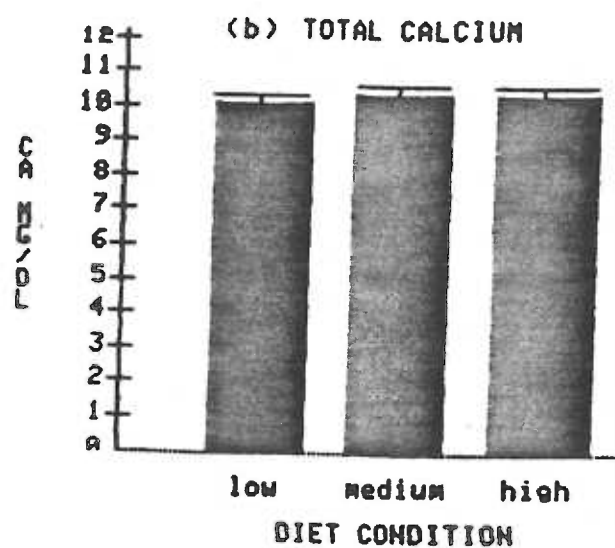
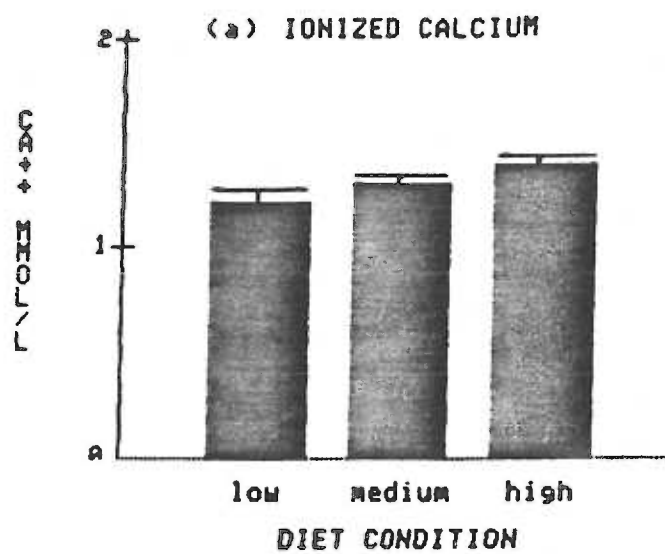
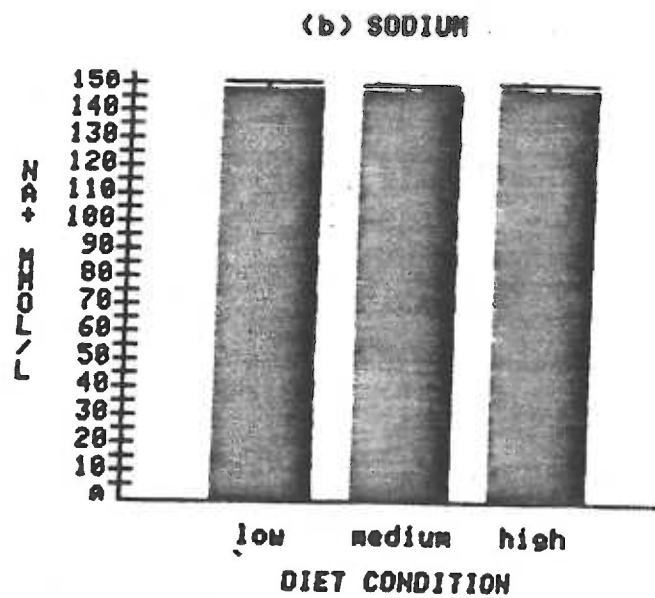
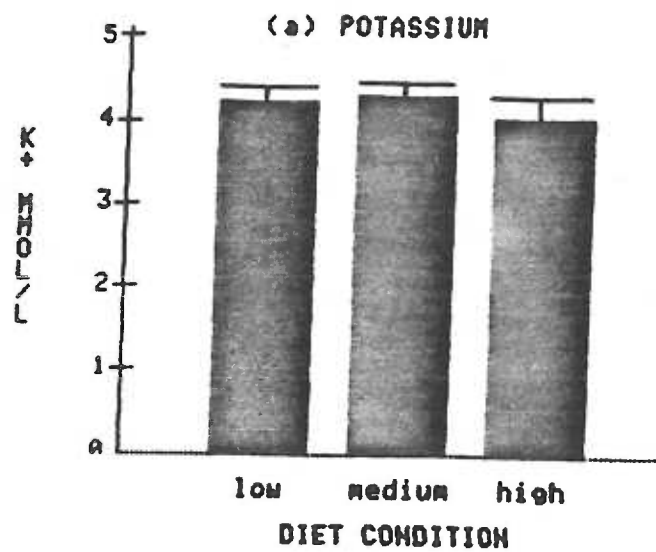


Figure 3. The effect of diet condition on mean (+S.D.) serum potassium (A) and sodium (B) in pre-pregnant SHR dams.



As indicated in Figure 3, high calcium dams experienced a small reduction in serum potassium ($F_{2,62} = 4.59, p < .05$). Subsequent Newman-Keuls tests revealed that the high calcium rats had significantly lower serum potassium levels than both the low ($p < .05$) and medium ($p < .01$) diet rats. The comparison between the low and medium diet rats was not significant. Manipulations of dietary calcium did not significantly effect serum sodium.

The prepregnant dams did not show a consistent blood pressure response to dietary calcium. These data are presented in Table 1. Although the medium group appeared to have lower pressures than the high and low groups, the overall analysis revealed no significant differences between diet conditions. Also presented in Table 1 are the pregnancy rates obtained from the dams on the three calcium diets.

The distribution of litters resulting from the fostering procedure are shown in Figure 4. An unusually low pregnancy rate (56%) in the medium calcium group necessitated a reduction in the cell sizes for litters per dietary group. The original design called for 8 litters per group assuming a pregnancy rate of 70%, however, the deficiency in the medium group reduced the litters to 7 in the low calcium group, 6 in the medium group and 6 in the high group. Except for the loss of one litter in the medium group, all pups survived to weaning.

Calcium had no effect on the maternal behaviors of the SHR dams. However, significant main effects for the time of day and the day of lactation were obtained for each of the behavioral parameters measured. These data are depicted in Figure 5.

The overall analysis for "time spent nursing" revealed significant main effects for the time of day and for the day of lactation.

Table 1. Maternal data after 6 weeks of dietary treatment
(12 weeks of age)

Diet	N	Mean ($\pm$ SD) SBP (mmHg)	Number of Dams Made Pregnant	Pregnancy Rate
Low	15	169.1 $\pm$ 24	10	67%
Medium	36	149.6 $\pm$ 28	19	53%
High	15	169.3 $\pm$ 15	13	87%

Figure 4. The distribution of litters resulting from the fostering procedure showing the number of litters that survived to weaning.

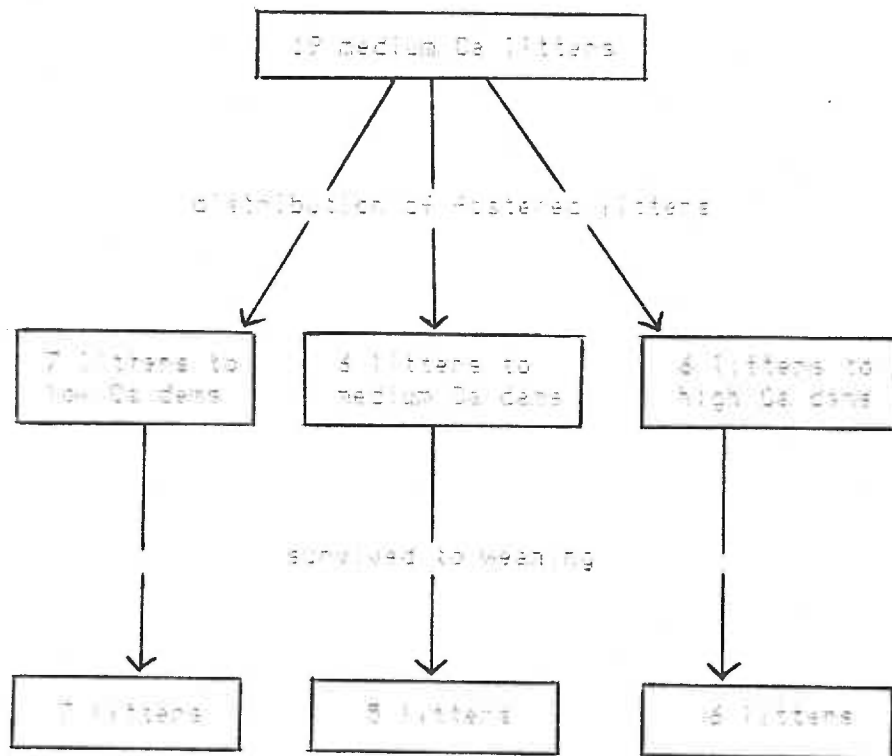
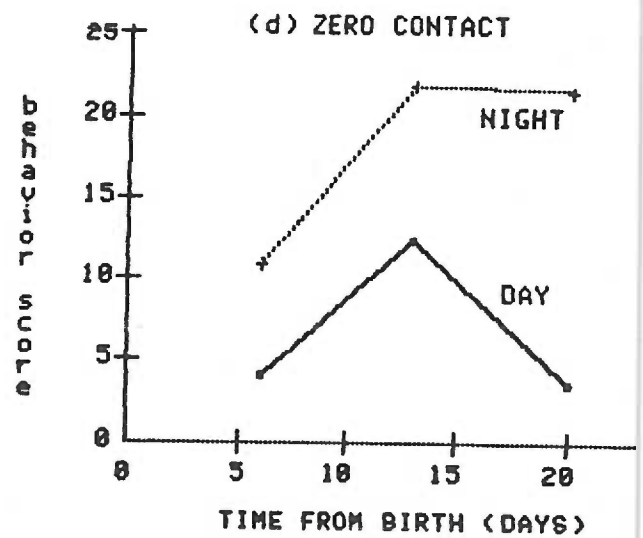
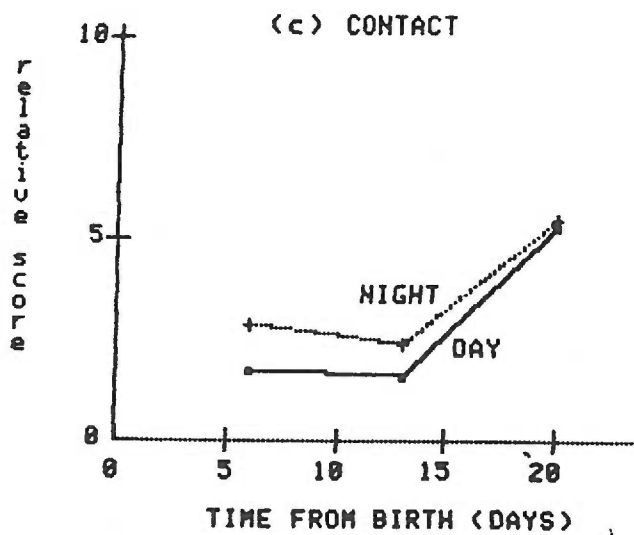
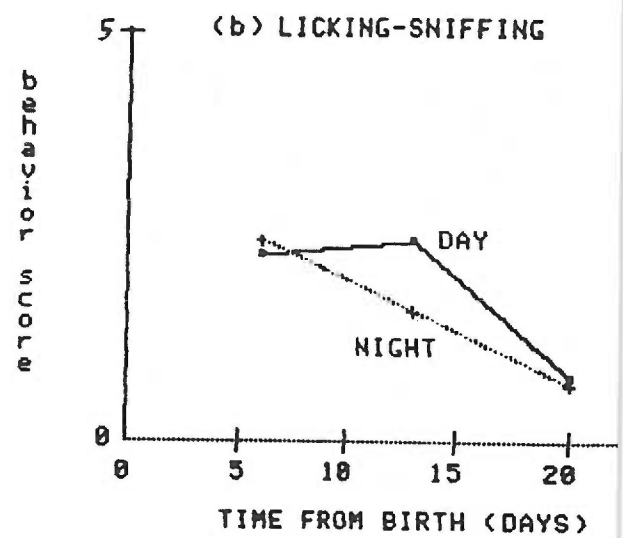
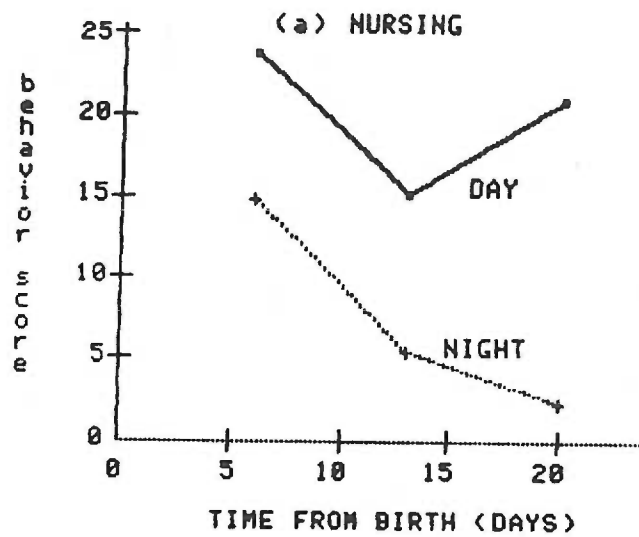


Figure 5. The time that SHR dams spend nursing (A), licking-sniffing (B), in simple contact (C), and in zero contact (D) with their pups during hours 1-2 pm (day) and 11-12 pm (night) on days 6, 13, and 20 of lactation , averaged across diet conditions. Each score represents the average number of times a particular behavior was recorded out of 30 observational periods.



Inspection of Figure 5A reveals that SHR dams spend considerably more time nursing during the day than during the night ($F_{1,15} = 36.91$, $p < .01$). The post hoc analysis of the days of lactation collapsed across the time of day showed that the dams spend more time nursing during day 6 of lactation than during either day 13 or day 20 ($p < .01$).

The SHR dams spent relatively more time licking and sniffing their pups early in lactation ($F_{2,30} = 5.53$, $p < .01$). Specifically, the post hoc analysis revealed that the dams tended to spend more time licking and sniffing pups during days 5 and 13 of lactation than during day 20. These differences approached but did not attain the $p = .05$ level of significance.

The amount of time that the dams spent in simple contact with their pups varied across days 6, 13, and 20 of lactation ($F_{2,30} = 8.75$, $p < .01$). As can be seen in Figure 5c, the time spent in simple contact appears to go up at day 20 of lactation. Subsequent analysis using Newman-Keuls tests show, however, that day 20 is statistically different from day 13 ($p < .05$) but not from day 6.

The analysis of time spent in zero contact with pups revealed a main effect for time of day ($F_{1,15} = 39.16$), for the days of lactation ($F_{2,30} = 12.84$, $p < .01$), and a time of day by days interaction ($F_{2,30} = 4.83$, $p < .05$). The effect for the time of day reflects more time spent by the SHR dams away from their pups during the night as compared to the day.

The zero contact seen across the days of lactation was best described by the time of day by days interaction. An inspection of Figure 5d suggests that the interaction was due to a decrease in zero contact during day 20 in the day time, whereas during the night, the amount of

zero contact remains elevated. To analyze the interaction, difference scores were computed by subtracting each subject's night score from it's day score. The overall analysis was made using a one way ANOVA comparing the difference scores as they varied across days 5, 13, and 20 of lactation. This analysis revealed a significant day of lactation effect ($F_{2,30} = 4.83, p < .05$), and the subsequent Neuman-Keuls tests revealed that day 20 difference scores were significantly larger than either 6 or day 13 ($p < .05$). The difference scores computed for days 6 and 13 did not differ from one another.

The blood chemistries for the 16 day old male SHR pups are presented in Figures 6 and 7. The data for ionized calcium was not available because the blood samples obtained did not provide an adequate volume for that assay. In addition, two samples, one from the medium condition and one from the low condition, were lost due to hemolysis during the sampling process. As is suggested in the figures, manipulations of dietary calcium in the dams had no effect on the serum electrolytes of the pups at sixteen days of age, and the analysis of variance of the data revealed no significant differences between any of the diet groups.

In contrast to the electrolyte data at 16 days, the pups at 28 days demonstrated nearly the same serum electrolyte response to dietary calcium manipulations as did the prepregnant dams. In general, pups suckling high calcium dams had higher serum calcium values and lower phosphorus and magnesium than pups assigned to low calcium dams. These data are depicted in Figures 8 and 9. Because the between group analysis of variance of the 28 day electrolyte and blood pressure data revealed no differences between the male and female pups, the following

Figure 6. The effect of maternal diet on mean ($\pm$ S.D.) serum total calcium (A), magnesium (B), and phosphorus (C) in 16 day old male SHR pups.

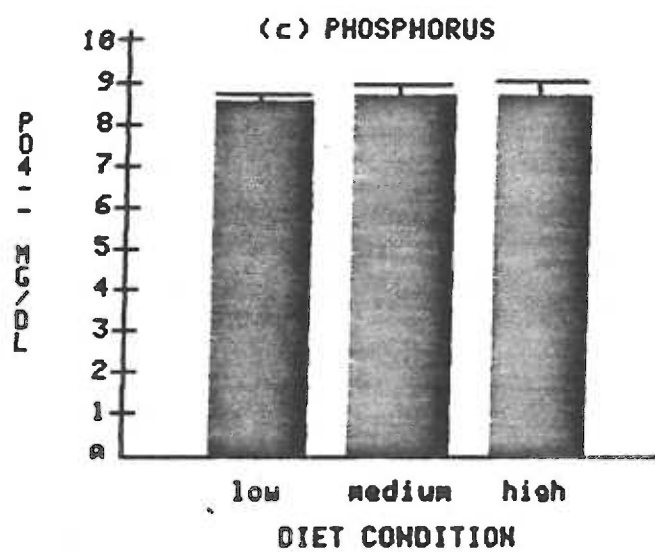
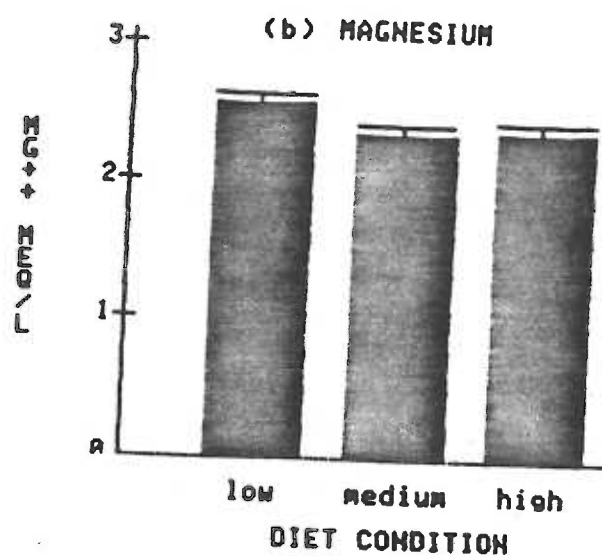
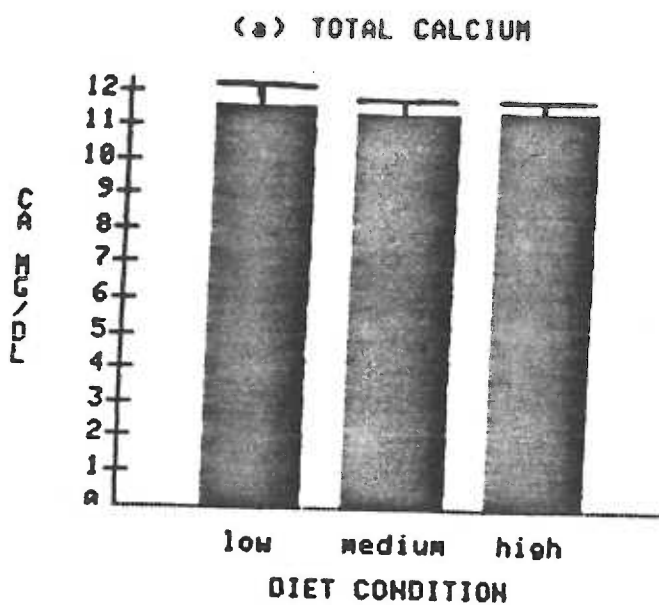
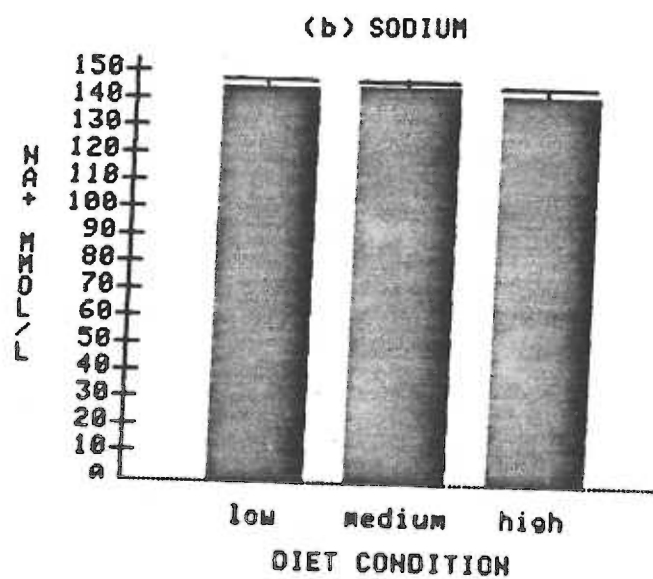
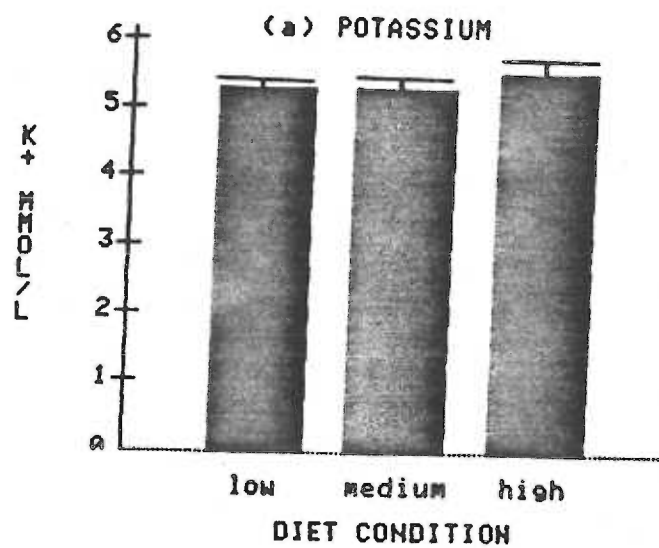


Figure 7. The effect of maternal diet on mean ($\pm$ S.D.) serum potassium (A) and sodium (B) in 16 day old male SHR pups.



analyses are collapsed across sex.

Diet had a significant effect on serum total calcium ($F_{2,30} = 6.79$, $p < .01$). The high calcium pups had significantly higher total calcium than was demonstrated in the other two conditions ($p < .01$). The overall ANOVA for ionized calcium showed a highly significant effect for diet ($F_{2,30} = 27.82$, $p < .01$), and the Newman-Keuls test showed that high calcium pups had the highest ionized calcium blood levels ($p < .01$), and the medium and low groups did not significantly differ from one another.

Serum phosphorus was inversely related to the level of dietary calcium ingested by the pups ($F_{2,30} = 22.14$, $p < .01$). Subsequent post hoc tests revealed that each of the diet conditions was significantly different from one another. Mean phosphorus levels differed greatly between the low and high groups and between the medium and high groups ($p < .01$), whereas the difference between the low and medium groups was smaller in magnitude ($p < .05$).

As with serum phosphorus, dietary calcium varied inversely with the levels of serum magnesium ($F_{2,30} = 46.49$, $p < .01$). The Newman-Keuls tests revealed that serum magnesium levels were different across each of the three diet conditions. The differences between the high and low and the medium and low groups attained the $p < .01$ level of significance, whereas the difference between the medium and high groups attained the $p < .05$ level of significance. Diet had no effect on either serum sodium or serum potassium.

At 28 days, pups on the low calcium diet had the highest blood pressures, whereas high calcium pups had the lowest ($F_{2,30} = 7.59$, $p < .01$). These data are depicted in Figure 10A. The differences in blood

Figure 8. The effect of maternal diet on mean ($\pm$ S.D.) serum ionized calcium (A), total calcium (B), magnesium (C), and phosphorus (D) in 28 day old SHR pups, averaged across sex.

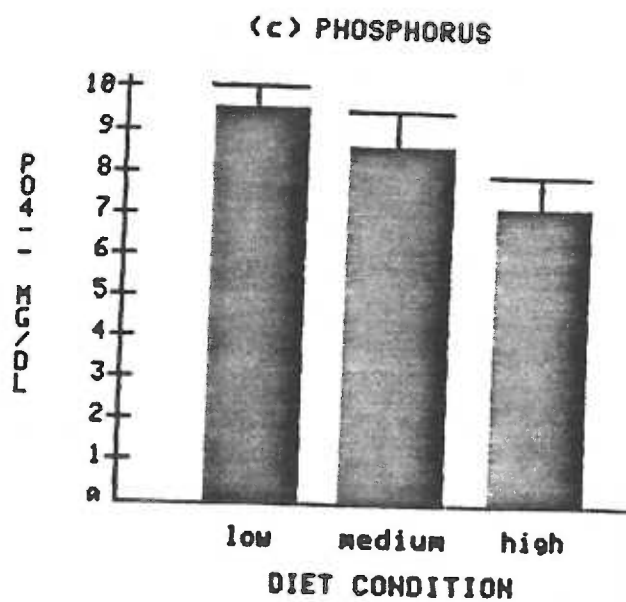
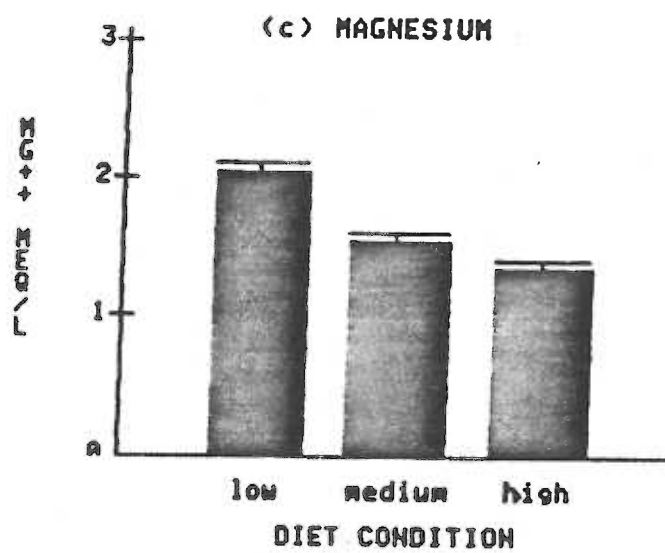
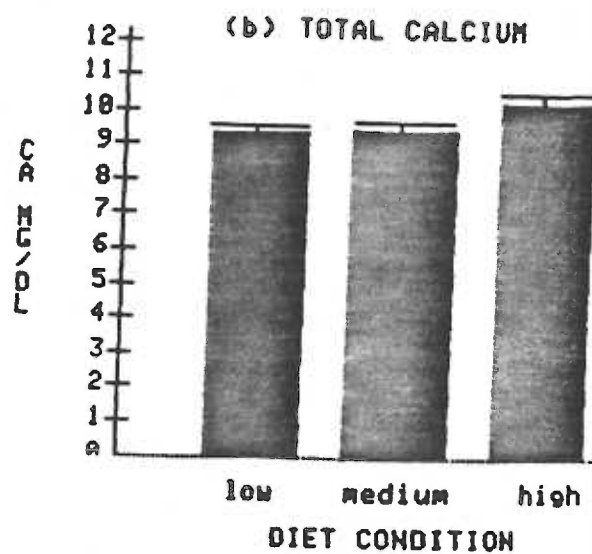
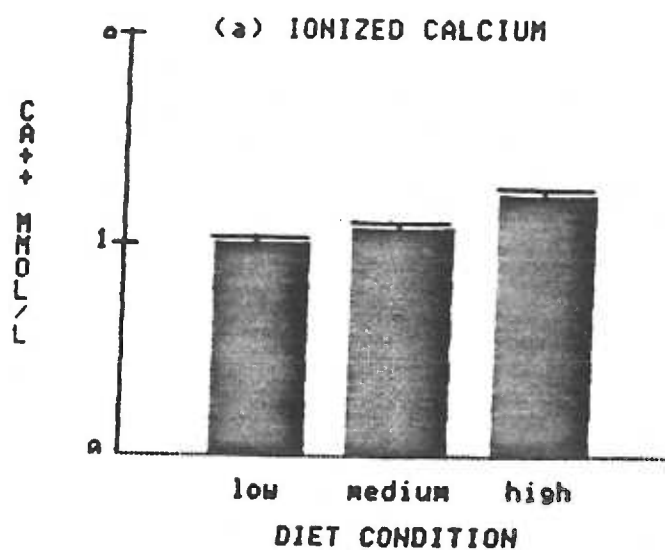


Figure 9. The effect of maternal diet on mean (+S.D.) serum potassium (A) and sodium (B) in 28 day old SHR pups, averaged across sex.

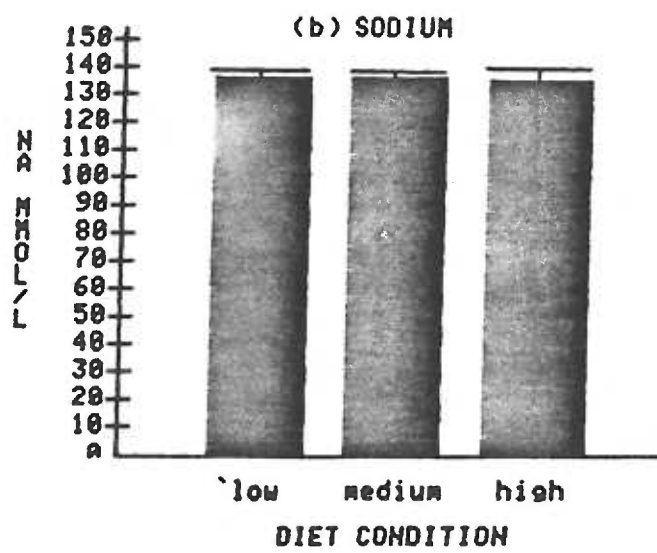
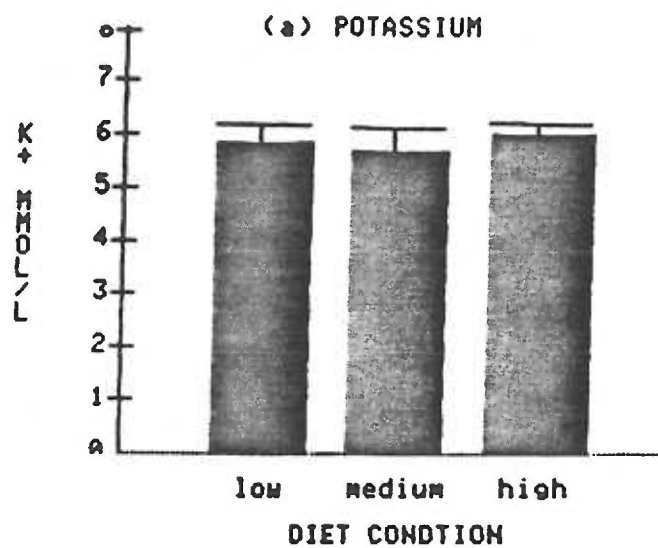
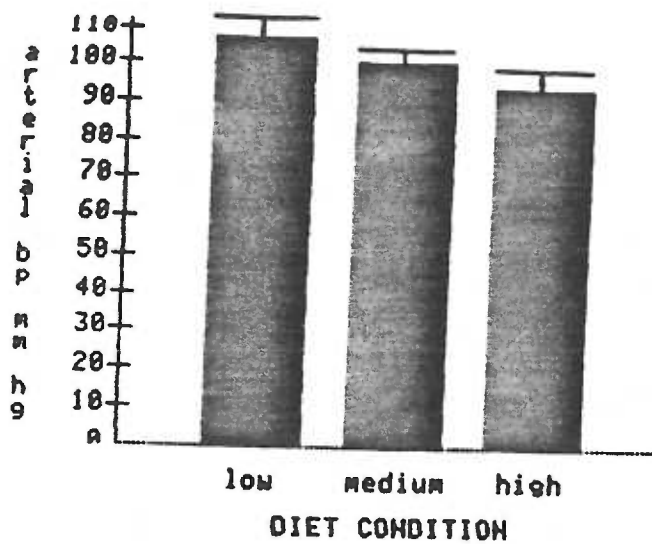
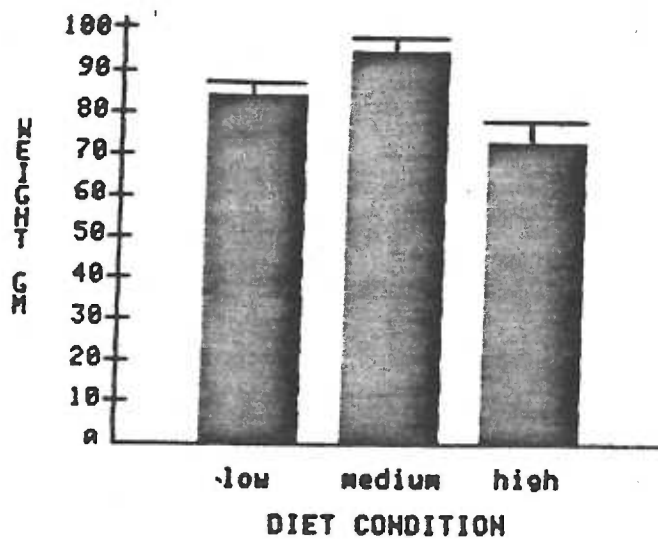


Figure 10. The effect of maternal diet on mean (+S.D.) arterial blood pressure (A) and weight (B) in 28 day old SHR pups, averaged across sex.

(a) WEANING BP



(b) PUP WEIGHTS AT WEANING

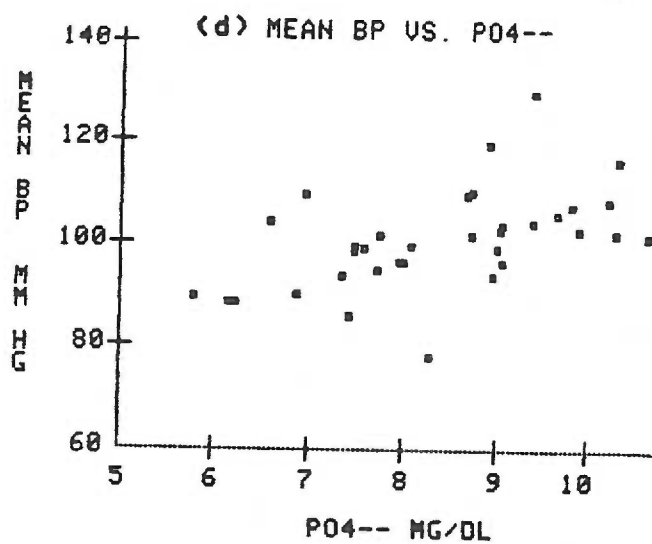
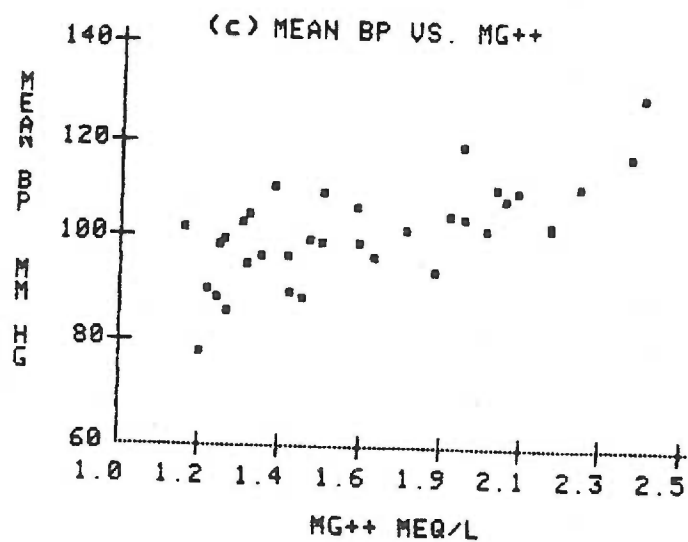
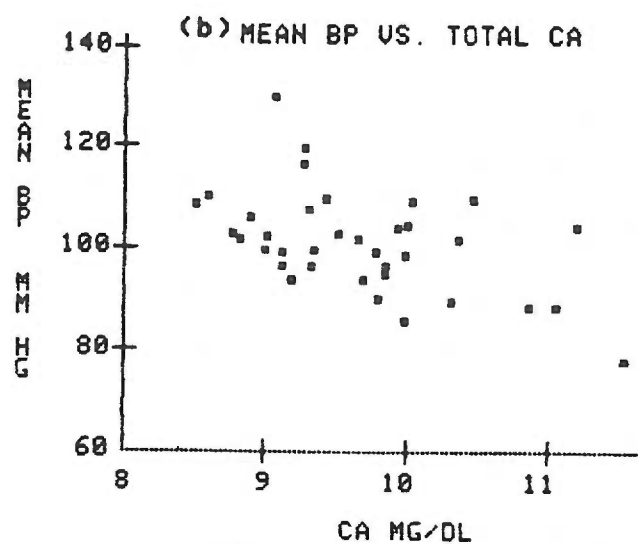
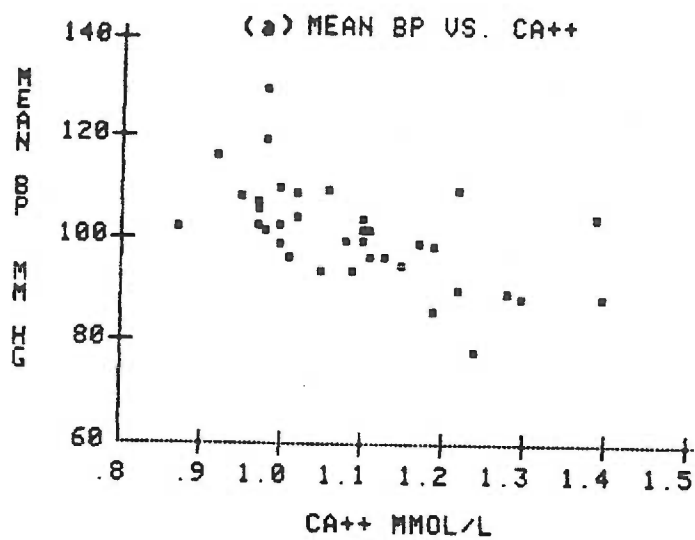


pressures were significant between the low and high conditions ($p < .01$) and between the low and medium conditions ($p < .05$), but not between the medium and high conditions ($p = .06$).

The comparison of pup weights at 28 days is depicted in Figure 10B. As can be seen in the figure, pups suckling high calcium dams had the lowest weights while medium calcium pups had the highest weights ($F_{2,34} = 10.10$, $p < .01$). Analysis of variance of weights confirmed this observation and subsequent Newman-Keuls tests demonstrated that the difference between the high and medium diets attained the $p < .01$ level of significance. The differences between the low and medium and the low and high conditions were significant at the $p < .05$ level.

The correlations of serum ionized and total calcium, magnesium, and phosphorus with mean arterial BP in the weanling pups are presented in Figure 11. A Pearson's r analysis of this data revealed that both serum ionized ($r(34) = -0.59$, $p < .01$) and total calcium ($r(34) = -0.50$, $p < .01$) were inversely related to mean arterial BP, whereas both magnesium ($r(34) = 0.67$, $p < .01$) and phosphorus ($r(34) = 0.54$, $p < .01$) demonstrated a positive relationship.

Figure 11. A scatter plot comparing blood pressure with serum ionized calcium (A), total calcium (B), magnesium (C), and phosphorus (D) for each pup at 28 days of age.



G. Discussion:

The results indicate that dietary calcium manipulations have a significant effect on blood pressure at weaning. Spontaneously hypertensive rat pups on the low calcium diet had the highest blood pressures, whereas high calcium pups had the lowest. Although this finding agrees with previous reports showing an inverse relation between dietary calcium and blood pressure in adult SHR_s (McCarron, 1981; 1984), this is the youngest age at which such a relationship has been demonstrated.

Two earlier experiments have examined the influence of dietary calcium on blood pressure in young SHR_s, however, neither study manipulated calcium before 4 weeks of age. For example, Stern (1984) placed SHR_s on normal and high calcium diets at 6 weeks of age. When blood pressures were measured 4 weeks later at 10 weeks of age, no differences were found between the two dietary groups. The other study found lower blood pressures in SHR_s begun on a high calcium diet at 4 weeks of age as compared to 10 weeks, however, the first systolic blood pressure measurement was not taken until both groups reached 15 weeks of age (McCarron, 1982b).

The alterations in pup blood pressures may have been induced by a milk borne hypertensinogenic factor transferred to the pups via suckling. To explore this possibility, the influence of dietary calcium on the electrolyte physiology of the dams was examined. It was reasoned that serum electrolyte alterations in the dams may have been reflected in

electrolyte or hormonal changes in the milk which could ultimately change the blood pressures of the pups.

The data demonstrate that increasing concentrations of calcium in the diet of the dams caused increased levels of serum calcium and decreased serum magnesium and phosphorus. As has been previously demonstrated (McCarron, 1981), the alterations in serum calcium result directly from the dietary calcium manipulations. Low serum magnesium, on the other hand, is a well known secondary response to chronic hypercalcemia (Parfitt, 1980). Calcium infusion greatly increases urinary magnesium excretion, and the effect has been attributed to impaired magnesium tubular reabsorption due to competition with calcium for the same transport mechanism (Parfitt, 1980). Conversely, low serum calcium reduces the competition for tubular reabsorption and causes an elevation in serum magnesium.

The low serum phosphorus associated with high dietary calcium can be accounted for primarily by an intestinal mechanism. Phosphate absorption across the intestine falls markedly with a high dietary calcium-to-phosphorus ratio thus creating a model for dietary phosphorus deprivation (Spencer, 1984). Furthermore, a rise in serum calcium increases the formation of CaHPO_4 ion-pair complexes which triggers a further fall in free serum phosphorus (Parfitt, 1980).

The altered serum electrolytes found in the females favor the possibility that similar alterations were reflected in the milk which in turn may have influenced the blood pressure of the pups by 28 days. However, a previous study demonstrated that rat pups begin supplementing their milk diet with solid food by about 16 days of age (Redman, 1976). Therefore, it is also possible that consumption of the different calcium

diets between 16 and 28 days may be directly responsible for the blood pressure alterations.

In an attempt to discover which dietary factors, milk or solid food, may have been more important in determining blood pressure in the pups, blood samples were drawn from the pups at 16 and 28 days for determination of serum electrolytes. The data show no alterations in electrolytes at 16 days, whereas at 28 days there were induced changes in serum ionized calcium, total calcium, phosphorus, and magnesium. Both total and ionized calcium were elevated at 28 days in pups suckling high calcium dams relative to pups suckling low calcium dams. Phosphorus and magnesium showed the reverse trend with the lowest levels found in pups suckling high calcium dams.

Whether the observed electrolyte alterations at 28 days were caused by milk or solid diet consumption cannot be determined from the electrolyte data because altered milk electrolytes may or may not have been reflected as altered serum electrolytes in the pups at 16 days. It is conceivable, for instance, that pups receiving altered milk electrolytes may have experienced cumulative changes in their electrolyte balance that were only detectable in their serum late in weaning.

The pattern of electrolyte changes at 28 days across the three suckling groups is similar to the prepregnant female data and suggests that the pups received varying amounts of calcium in their neonatal diet. Specifically, pups assigned to high calcium dams showed a pattern of electrolytes consistent with a high calcium diet, whereas pups assigned to low calcium dams apparently received a diet low in calcium. As was suggested above, however, it is not clear whether the pups received differing calcium diets as a consequence of their milk diets,

or as a result of their solid diets.

Previous results which favor a milk mechanism demonstrated that dietary calcium modifications can directly modify both serum calcium and the calcium content of the milk in lactating rats (Fairney, 1970). Although milk calcium content was not measured in the present experiment, it can be hypothesized from Fairney's study that dams on the high calcium diet may have produced a milk high in calcium. The pups suckling these dams may therefore have developed high serum calcium and depressed phosphorus and magnesium as a consequence of their high milk calcium.

Favoring a solid diet mechanism is the previously stated finding that solid diet consumption becomes increasingly important between 16 and 28 days. These data suggest that solid diet consumption could independently account for the 28 day results. For example, pups suckling high calcium dams may have developed high serum calcium and depressed phosphorus and magnesium simply as a result of the high solid calcium diet they consumed.

The amount of milk production may also have been influenced by the maternal calcium diets. This possibility was suggested by a study which demonstrated that vitamin D deficient rats produced smaller quantities of milk (Brommage, 1984). Similarly, dams on the low calcium diet may have produced reduced quantities of milk throughout lactation, and pups suckling these dams may have in turn received a reduced net quantity of calcium. This mechanism could independently account for the low serum calcium seen in these pups. Given this hypothesis, it might be expected that pups receiving the smallest quantities of milk should weigh the least at weaning, whereas the high calcium pups should weigh the most

due to their presumably higher milk intake. The weight data show, however, that the high calcium pups actually weighed the least while the medium calcium pups had the highest weights at weaning.

The alterations in serum calcium in the pups, whether caused by milk or solid diet, may have been directly responsible for the blood pressure alterations at 28 days. Serum calcium is suspected to have direct effects on the contractility of vascular smooth muscle. The higher levels of serum calcium in pups suckling high calcium dams may stabilize the vascular smooth muscle and lead to the lower blood pressures observed in this group (McCarron, 1984). The low serum calcium, found in pups assigned to the low calcium dams, on the other hand, is thought to increase the excitability of smooth muscle and increase blood pressure (McCarron, 1981).

An alternative explanation can be found in the phosphorus data. The intestinal absorption of phosphorus is known to be inhibited by high dietary calcium. Therefore, pups receiving a diet high in calcium experienced low serum phosphorus as a consequence of the high intestinal calcium. Because low serum phosphorus has been linked to hypotension (Lau, 1984), the lower blood pressure of the high calcium pups may in fact be due to their reduced phosphorus levels. Recent studies have shown that phosphate depletion is associated with numerous biochemical derangements in myocardial cells, resulting in an impaired production of energy in the heart. Chronic phosphate depletion is therefore thought to exert its hypotensive actions via a pathophysiologic reduction in cardiac output (Saglikes, 1985).

Serum magnesium was also altered in the pups as a consequence of the calcium in the diet. The hypothesis that altered magnesium may have

induced the blood pressure changes at weaning is unlikely because the direction of change is opposite from that which is normally expected for magnesium manipulations. For example, high calcium diet pups experienced low serum magnesium and lower blood pressures. Numerous studies have reported, however, that magnesium supplementation rather than depletion results in lower blood pressure via a relaxation of the smooth muscle membrane (Berthelot, 1983; Resnick, 1984).

Some other factor in the mother's milk, besides calcium, may be responsible for the blood pressure alterations in the pups. For example, the changes in serum electrolytes observed in the dams may have resulted in hormonal alterations that were reflected as differing hormonal concentrations in the milk. Hormones that respond to changes in serum calcium include serum PTH (Spencer, 1984) and renin (Brinton, 1975). Alterations in 1,25 dihydroxyvitamin D₃ synthesis would be expected to follow the calcium induced changes in serum PTH (Brommage, 1985). Of these hormones, PTH is known to have a direct effect on blood pressure (Schleiffer, 1978), and renin an indirect effect via angiotensin II formation (Eckert, 1978). Therefore both PTH and renin could presumably influence the blood pressure of the pups through a milk transfer mechanism. The intestinal absorption of these two proteins is doubtful, however, because of the probable enzymatic degradation that occurs in the gut (Eckert, 1978).

Although 1,25 dihydroxyvitamin D₃ has no demonstrable blood pressure effects, it could potentially influence the calcium metabolism of the pups and thus alter their blood pressure indirectly. For example, the low calcium dams may have experienced a reflexive rise in serum PTH which would initiate an increased production of 1,25 dihydroxyvitamin

D3 (Leheninger, 1982). A corresponding elevation in the milk levels of 1,25 dihydroxyvitamin D3 could serve to increase the calcium absorption and thus the serum calcium levels of the pups suckling these dams (Brommage, 1984). The results, however, do not support this hypothesis because pups suckling low calcium dams experienced low serum calcium rather than the predicted hypercalcemia. Furthermore, Dostal (1983) has demonstrated that alterations of 1,25 dihydroxyvitamin D3 in lactating dams do not alter the calcium metabolism of pups suckling those dams. The authors concluded that this hormone was not readily transferred through the milk.

Alternatively, it was possible that the atrial natriuretic factor and the natriuretic hormone may have been released in response to blood pressure changes in the dams. These two hormones may therefore have been differentially transferred to the pups where they may have had an influence on blood pressure. Although the prepregnancy pressure determinations revealed no differences, blood pressure may have changed during pregnancy and lactation. Dietary calcium induced alterations in blood pressure would seem more likely during this period because of the great demands that pregnancy and lactation place on calcium metabolism. Belizan (1981) has reported, however, that the blood pressure response to dietary calcium in female rats is not altered by pregnancy. It was additionally possible that the release of these two hormones may have been influenced by a direct action of calcium on cell membrane function. The alterations of extracellular calcium in the dams may have influenced the membrane stabilization of the tissues controlling the secretion of the atrial natriuretic factor and the natriuretic hormone. The releasing activity of these tissues may therefore have been influenced

by the calcium diets (Kwan, 1985).

There is some question as to whether the dietary calcium induced alterations in pressure represented chronic alterations in blood pressure or only acute responses to stressful stimuli. It will be recalled that blood pressure was measured from animals under loose restraint, which has been shown to be a powerful behavioral stressor in previous studies (Kvetnansky, 1979). Recently, Hatton et al (1986) have demonstrated that dietary calcium influences the acute blood pressure response of SHRs to a tone signalling an aversive stimulus during classical conditioning. This finding suggests that the pressure differences in the present study may only represent the acute blood pressure responses of the pups to the restraint stress.

Pup weights at weaning represent yet another diet induced alteration that may be related to blood pressure. A number of reports have linked obesity with an increased risk of becoming hypertensive (Horan, 1985). Although the present study associated lower blood pressures with low weight in the high calcium diet group, the weights of the medium and low calcium groups did not follow blood pressure in the expected manner. Rather, the highest weights were found in the medium group which displayed intermediate blood pressures at weaning. Therefore, it was concluded that other factors besides weight change were additionally responsible for the alterations in pup blood pressure.

The alterations of dietary calcium in the dams may have influenced their maternal behaviors which in turn may have altered the blood pressure of the pups. A behaviorally induced change in blood pressure is especially likely in SHRs in light of their exaggerated pressor responses to environmental stimuli (Trippodo, 1981). Because of these

possibilities, observations were made on the maternal behaviors of the dams on days 6, 13, and 20 postpartum. Behaviors such as nursing and licking-sniffing were chosen because they involved direct interactions between the dams and their pups. The observations were intended to detect any gross behavioral changes that may have occurred as a consequence of the calcium diets.

The results did not reveal any behavioral differences between the dams across the three diet conditions. On the basis of this finding, it was unlikely that maternal behavior had a differential effect on pup blood pressure. The general findings of increased independent behavior by the dams during the night, and a decrease in maternal attention to the pups with the approach of weaning are in agreement with the literature on rat maternal behavior (Priestnall, 1983; Dennenberg, 1967).

A further comment should be made about the blood pressure differences observed in the pups and adults. Although both male and female pups displayed blood pressure responses to the calcium manipulations, the same calcium diets had no blood pressure effect on the adult prepregnant females. This is in contrast with other reports that have utilized the same levels of dietary calcium and demonstrated strong blood pressure responses in adult male SHR_s (McCarron, 1984). Previous investigators have demonstrated blood pressure alterations with dietary calcium in female Wistar rats (Belizan, 1981) and in female SHR_s (Lau, 1984), however, the dietary manipulations in these studies were extreme compared to the present experiment. Nevertheless, adult female SHR_s are apparently more resistant than males to the cardiovascular effects of dietary calcium. This difference suggests that some aspect of the mature female physiology may be responsible. One possibility is

suggested by the elevated estrogen levels which correlate with the onset of puberty in rats (Eckstein, 1973). Estrogen is known to directly affect bone resorption of calcium (Worthington-Roberts, 1985) and is suspected to have an influence on 1,25 dihydroxyvitamin D3 metabolism (Gray, 1982). Whether endogenous estrogen in SHR's has any influence on the blood pressure response to dietary calcium remains to be determined.

In regards to electrolyte alterations, it should be recalled that the pre-pregnant females showed nearly the same calcium, phosphorus, and magnesium changes as the 28 day old pups. This finding suggests again, that adult female SHR's may somehow be resistant to the blood pressure altering abilities of these electrolytes, or that alterations in serum calcium, phosphorus, and magnesium per se have little effect on blood pressure.

H. Summary-Conclusions

The purpose of the present experiment was to determine the influence of dietary calcium on the development of blood pressure in neonatal spontaneously hypertensive rats. Adult SHR dams were placed on low, medium, and high calcium diets. After 6 weeks on the diets, indirect blood pressure was taken and blood samples were assayed for serum electrolytes. Shortly thereafter, the dams were bred. The offspring from normal calcium females were given to foster mothers on the low, medium, and high calcium diet conditions. Blood samples were drawn from the pups at 16 and again at 28 days for determination of serum electrolytes. Mean arterial blood pressure was measured in the pups at 28 days using an indwelling catheter. In addition, the dams on the three calcium conditions were observed on days 6, 13, and 20 postpartum for diet induced alterations in maternal behavior.

The principal finding was that dietary calcium had a pronounced effect on blood pressure at weaning. Pups suckling low calcium dams had the highest pressures while high calcium pups developed the lowest pressures. At 16 days of age, the pups showed no dietary induced alterations in serum electrolytes, however, by 28 days it was found that pups suckling high calcium dams developed high serum total and ionized calcium and low phosphorus and magnesium. Low calcium pups, on the other hand, displayed low calcium and high phosphorus and magnesium. While the prepregnant females also responded to higher dietary calcium with increased serum ionized calcium and decreased phosphorus and

magnesium, they did not show any corresponding changes in blood pressure. The maternal behaviors observed were not altered by the dietary calcium manipulations.

The results indicate that early dietary calcium has a substantial impact on mean arterial blood pressure in SHR_s. A number of factors including altered milk electrolytes and milk hormones as well as late lactational solid diet consumption may have been responsible for the blood pressure alterations. Although the present results support some of the above hypotheses, further research will be needed to determine which, if any, are the principal cause of the blood pressure alterations seen in the pups.

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