IN VIVO AND IN VITRO STUDIES OF CARDIOCYTES IN GENETIC HYPERTENSION

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Department: Department of Medicine,
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Abstract: Comparative studies of organs from the spontaneously hypertensive rat (SHR) and the normotensive control rat Wistar Kyoto (WKY) have shown that the heart and kidney are enlarged at birth in SHR. Higher deoxyribonucleic acid (DNA) concentrations reflect cardiac and renal hyperplasia in SHR. We have estimated the rate of DNA synthesis with methyl-^{3}H-thymidine (^{3}H-TDR) labelling *in vivo* for newborn WKY and SHR organs. Most significant was the finding of a higher DNA specific activity for SHR heart (+33%; $p<0.05$) and kidney (+39%; $p<0.001$). *In vitro* incorporation of ^{3}H-TDR into DNA, autoradiography, cell number and flow cytometric analysis have shown similar cell size, cell cycle length, and DNA content per cell for newborn WKY and SHR cardiocytes. These findings support the hypothesis that in SHR the higher DNA content and specific activity *in vivo* are due to a greater number of "normally" growing cardiocytes. Altered beating rates, lower plating efficiency and higher thermolability represented additional genetic abnormalities of SHR cardiocytes.

ETUDES IN VIVO ET IN VITRO DES CARDIOCYTES DANS L'HYPERTENSION GENETIQUE

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Résumé: Il a été démontré que le coeur et les reins de rats spontanément hypertendus (SHR) sont hypertrophiés par rapport aux organes des rats témoins (WKY) à la naissance. L'augmentation de la concentration en ADN chez les rats spontanément hypertendus reflète une hyperplasie cardiaque et rénale. L'évaluation du taux de synthèse de l'ADN, dans les organes est effectuée par marquage *in vivo* avec de la thymidine tritiée. Nous avons remarqué une augmentation significative de l'activité spécifique de l'ADN au niveau du coeur (+ 33%, $p<0.05$) et des reins (+ 39%, $p<0.001$) chez les rats spontanément hypertendus. L'incorporation *in vitro* de la thymidine tritiée dans l'ADN, l'autoradiographie, le comptage cellulaire et l'analyse cytométrique ont démontré une similitude dans la longueur du cycle cellulaire, la taille des cellules, et leur contenu en ADN entre les cardiocytes des rats témoins et de ceux des rats spontanément hypertendus. Ces résultats supportent l'hypothèse qu'une

augmentation, à la fois du contenu en ADN et de l'activité spécifique *in vivo* chez le rats spontanément hypertendus, est dûe à un plus grand nombre de la population normale des cardiocytes. Par ailleurs, une modification de la fréquence des battements, une diminution de l'efficacité de mise en culture, et une thermolabilité accrue des cardiocytes représentent des anomalies génétiques additionnelles chez les rats spontanément hypertendus.

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To my parents, Alice and Charles

To my husband, Steven

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LIST OF ABBREVIATIONS AND UNITS

ADP	:	adenosine diphosphate
ATP	:	adenosine triphosphate
BAIB	:	β-aminoisobutyric acid
BSA	:	bovine serum albumin
BSS	:	buffered saline solution
BUIB	:	β-ureidoisobutyric acid
°C	:	degree celsius
cAMP	:	cyclic 3', 5' adenosine monophosphate
Ci	:	curie
CM	:	carboxy methyl
cm^2	:	square centimeter
CPK	:	creatine phosphokinase
CS	:	calf serum
cpm	:	counts per minute
DHT	:	dihydrothymine
DME	:	Dulbecco's modified Eagle's medium
DNA	:	deoxyribonucleic acid
DOCA-salt	:	deoxycorticosterone and salt induced hypertension
dpm	:	disintegration per minute
EDTA	:	disodium ethylenediaminetetra-acetate
EGF	:	epidermal growth factor
F12	:	Ham's F12 medium
FCS	:	fetal calf serum
g	:	gram

G	:	gravitational acceleration
G_0	:	gap 0 of cell cycle
G_1	:	gap 1 of cell cycle
G_2	:	gap 2 of cell cycle
H	:	histone
HBP	:	high blood pressure
^{3}H-DNA	:	methyl-^{3}H-thymidine incorporated into DNA
HEPES	:	N-2-hydroxypiperazine-N-2-ethane sulfonic acid
hr	:	hour
HSE	:	heat shock regulatory element
hsp	:	heat shock protein
HSTF	:	heat shock transcriptional factor
^{3}H-TDR	:	methyl-^{3}H-thymidine
^{3}H-Total	:	total methyl-^{3}H-thymidine
IGF	:	insulin-like growth factor
i.p.	:	intraperitoneal
kDa	:	kilodalton
mRNA	:	messenger ribonucleic acid
min	:	minute
mmHG	:	millimeter of mercury
NGF	:	nerve growth factor
NIH	:	National Institute of Health
ns	:	non significant at the level of 95%
OSF	:	oocyte-specific factor
p	:	probability

PBS	:	phosphate buffered saline
PCA	:	perchloric acid
PCS	:	phase combining system scintillation fluid
PDGF	:	platelet derived growth factor
PDS	:	plasma derived serum
PDS-CM	:	human plasma derived serum purified on CM-Sephadex
PE	:	platelet extract
PGF	:	polypeptide growth factor
pO_2	:	partial pressure of oxygen
pH	:	negative logarithm of hydrogen ion concentration
RNA	:	ribonucleic acid
SD	:	standard deviation
SDR	:	Sprague Dawley rat
SE	:	standard error
SHR	:	spontaneously hypertensive rat
Sp	:	stroke prone
Sr	:	stroke resistant
TDP	:	thymidine diphosphate
TDR	:	thymidine
TGF	:	transforming growth factor
TMP	:	thymidine monophosphate
TTP	:	thymidine triphosphate
TRIS	:	tris(hydroxymethyl)aminomethane
VSMC	:	vascular smooth muscle cells

WAR	:	Wistar rat
WBS	:	Whole blood serum
WKY	:	Wistar Kyoto rat

I - INTRODUCTION

The significance of cardiac hypertrophy associated with a genetic predisposition for hypertension is a controversial issue. Whether cardiac hypertrophy is antecedent to or concomitant with the rise in blood pressure, and to what extent the heart plays a role in the pathogenesis of the disease, are important questions that remain unanswered. Studies with the spontaneously hypertensive rat (SHR), a research model used extensively to further our understanding of human essential hypertension, have been designed to help clarify this association. Findings from several studies have led investigators to suggest that cardiac hypertrophy in SHR may be a primary genetic defect, and not just a secondary adaptive response to elevated blood pressure. The evidence supporting this concept includes: the presence of cardiac enlargement at birth in SHR; and the dissociation between the efficacy of certain antihypertensive drugs to lower blood pressure and heart rate, and their effect on cardiac hypertrophy.

Comparative studies, completed by Hamet et al[1] at the Clinical Research Institute of Montreal, of hearts, kidneys, and livers from newborn SHR and normotensive Wistar Kyoto rats (WKY) showed significantly higher relative protein and DNA contents (mg/g body weight) in SHR heart and kidney but not liver. The higher DNA contents provided evidence of cardiac and renal cellular hyperplasia reflecting a growth abnormality in newborn SHR heart and kidney.

The work presented in this thesis was undertaken to search for alterations in cardiac growth in newborn SHR. The possibility of a genetic growth defect and the presence of circulating growth factors in SHR with enhanced mitogenic potential were considered.

Completion of this work involved several approaches: *in vivo* incorporation of methyl-^{3}H-thymidine (^{3}H-TDR) into DNA of organs from WKY and SHR; growth rates determined by cell number of cardiocytes in culture; *in vitro* incorporation of ^{3}H-TDR into DNA; flow cytometric analysis of WKY and SHR cardiocytes; autoradiography; and preparation of blood components from WKY and SHR adults for evaluation of their growth stimulating potential. Other parameters such as heat sensitivity, beating rate, and plating efficiency, were also compared for WKY and SHR cardiocytes.

The present study is concerned with the relationships between several vast and complex areas of study, that of hypertension, cardiac growth, and heat stress. Therefore, only the investigations with a potentially interrelated significance are discussed in the review of the literature. Brief reviews of the cell cycle and thymidine are also included to provide enough background information for the procedures used.

II - REVIEW OF THE LITERATURE

A - ESSENTIAL HYPERTENSION

1 - DEFINITION

Essential hypertension is a disease defined by raised blood pressure without any evident cause[2]. Although essential hypertension is usually manifest as a disease of adulthood, current epidemiological studies confirm a long developmental period that starts in young adulthood or childhood and has even been detected in infancy[3,4]. Due to its early appearance in some children, and the high incidence in some families and ethnic groups, an inherited predisposition for essential hypertension has been suggested. The findings of a familial resemblance in blood pressure among children and their biological parents and siblings, but not among adopted children sharing the same home environment, confirms the genetic component[5,6].

Environmental factors such as physical and emotional stress, and salt intake are also known to play a significant role in determining the severity, and possibly the time of onset of the disease[7]. Because the stage of overt symptoms tends to occur mostly in the middle years, the early period of hypertension onset and development have not been studied as extensively as the established phase of the disease.

2 - RESEARCH MODELS FOR ESSENTIAL HYPERTENSION

Adequate animal models for human diseases are indispensable for the experimental study of their pathogenesis. Establishing animal models for genetic diseases, unlike infectious and nutritional disorders, is particularly difficult and many research models remain to be clarified in spite of extensive studies[8]. Essential hypertension had long been a typical example of such diseases for which animal models were not available until the late 1950's and early 1960's when the genetic models of hypertension in rats were independently developed in New Zealand[9], Japan[10], and the United States[11]. In the following two decades further models of hypertension were successively established[9-22], and artificially induced experimental hypertensive models such as renal and deoxycorticosterone-salt (DOCA-salt) hypertension, which had been preponderantly used for hypertension research, continue to serve as models of secondary hypertension.

B - THE SPONTANEOUSLY HYPERTENSIVE RAT

1 - STRAIN ESTABLISHMENT

The most frequently used animal model of genetically based hypertension is the spontaneously hypertensive rat (SHR)[10] which was developed by Okamoto and Aoki in Kyoto, Japan, in 1963. To establish this strain one male rat with spontaneous hypertension (150-175 mmHg) and one female rat with slightly higher blood pressure than the average (130-140 mmHg) were selected from a colony of Wistar rats bred at the Kyoto University. Experimental successive mating of brother and sister animals that maintained hypertension (blood pressure exceeding 150 mmHg persisting over a month) were carried out systematically. After the F3 generation these animals developed hypertension spontaneously as early as a few months after birth and were highly prone to develop severe hypertension with high frequencies of various hypertensive pathological lesions (e.g., stroke, myocardial fibrosis, and angionecrosis).

2 - THE SHR AS AN APPROPRIATE RESEARCH MODEL

The SHR is hemodynamically similar in many respects to human essential hypertension and has become the animal model of choice[23]. Its acceptance is based on the following criteria:

a) Hypertension in the SHR develops without the intervention of either surgery or other manipulatory practices (e.g., Dahl salt-sensitive strain and Sabra hypertensive strain need salt loading or DOCA-salt administration plus salt loading to produce hypertension).

b) Hypertension in the SHR has a hereditary component and does not appear to be of primary renal or simple neurogenic origin.

c) The SHR appears to be a sensitive experimental model for evaluating antihypertensive drugs.

d) The sequelae of the hypertension in the SHR resemble those seen in essential hypertension such as cerebral damage, arteriosclerosis, cardiac hypertrophy.

e) The SHR are generally available making it possible to compare the results of investigators in different laboratories.

In addition, the early development of hypertension initially noted in the SHR, where genetic factors are concentrated by the long-term repetition of selective breeding, can also be noted in humans[5,6]. Cardiac hypertrophy in SHR is present at birth and has also been observed in adolescents with borderline hypertension[24], and also in the offspring of hypertensive parents[25].

The use of the SHR as a model for essential hypertension has not been completely immune to criticism, obviously the genetic basis for hypertension in the SHR is more firmly established than for humans because the strain was specifically bred by brother-sister pairing of animals with higher than normal pressure, and since all individuals of the F3 generation of the strain developed high sustained pressure. Further criticism comes from the lack of a proper normotensive control strain for this model. The original breeding stock of Wistar Kyoto (WKY) was not maintained in parallel to that of the SHR and animals with blood pressure at the upper limits were usually withdrawn from the WKY breeding program. Therefore, the control animals may have a somewhat lower blood pressure than "normal" making interpretations of significant differences between WKY and SHR somewhat confusing as to whether they are the result of "hypertensive genes" in SHR or "low pressure genes" in WKY. Furthermore, differences observed between WKY and SHR may be due to a chance selection of genes unrelated to the regulation of blood pressure[26].

When weighing the pros and cons of SHR as an appropriate research model it is generally agreed that the SHR is important not only for studies on the pathogenesis and therapy but also the prevention and prognosis of hypertensive diseases, studies which are expected to contribute to the preventative treatment of hypertension and the related complications.

The number of studies of the neonatal manifestations of hypertension in SHR has increased in recent years, and most have been undertaken with the object of answering questions regarding which hypertensive characteristics are genetically determined and therefore accompany or cause the pressure rise (primary defect), which ones are environmentally caused, and which ones are adaptive or result from the pressure rise (secondary defect). We have recently reviewed research strategies to distinguish between primary and secondary changes in essential hypertension[26].

C - CARDIAC GROWTH AND HYPERTROPHY

1 - THE GROWTH RESPONSE

The anatomical and functional aspects of cardiac development in health and disease have been reviewed in detail[27]. The growth response of the heart can take several forms[28]: (a) cardiac enlargement (cardiac hypertrophy) which can be accomplished either by proliferation of heart cells (cellular hyperplasia), enlargement of existing cells (cellular hypertrophy) or a combination of both processes; (b) change in the relative amounts of cellular constituents, such as preferential synthesis and accumulation of mitochondria in the phase of developing hypertrophy; and (c) change in the complement of cellular proteins, such as replacement of one myosin isozyme by another. The form(s) of gene activation which participate in the cardiac growth response will depend in part on the nature and extent of the physiological demand and also the age of the animal.

Evidence of age-related factors influencing the adaptive response of the heart comes from experimental procedures designed to produce volume or pressure overload in young and adult animals. In young rats[29,30], cardiac enlargement was a result of muscle and non-muscle cell hyperplasia as demonstrated by incorporation of ^{3}H-TDR into muscle nuclei. On the other hand, induced cardiac enlargement in mature animals[29-32] was primarily due to enlargement of pre-existing muscle cells (hypertrophy) and non-muscle cell proliferation (hyperplasia). The response of these cells to growth stimuli depends largely on their ability to synthesize DNA at the time at which the stimulus is applied. Selective activation of cell division must also occur, since non-muscle cell DNA synthesis was observed in hearts of both young and adult animals during overload stress[28].

In a study designed to observe the transition from hyperplastic to hypertrophic myocardial cell growth during normal development, labelling indices were determined from autoradiographs of perfused fixed hearts of newborn to 21 day-old Fischer 344 rats which had received an intraperitoneal injection of ^{3}H-TDR[33]. From this experiment, three phases of postnatal myocardiocyte growth were suggested. In the first phase, birth to 4 days of age, there was a sustained hyperplastic growth of myocardiocytes. During this time period, myocardiocytes continued to synthesize DNA, decreased ^{3}H-TDR/cell by 50%, and fewer than 4% were binucleated. A second phase of growth was observed from 6 to 14 days of age and represented a transition from hyperplastic to hypertrophic growth, although the nuclei of myocardiocytes were continuing to synthesize DNA, there was a significant decrease in number of labelled nuclei, similar to the reports of others[34,35]. There was also a rapid transition from mono- to binucleated myocardiocytes. The third phase, 14 to 21 days of age, represented hypertrophic growth characterized by a continued increase in heart weight, little DNA synthesis, no change in percentage of binucleated cells and no change in dpm/cell. From these findings, it was also suggested that the formation of binucleated myocardial cells may be an early indicator of growth hypertrophy in young rats and possibly a result of mitosis without cytokinesis.

2 - FACTORS INFLUENCING CARDIAC GROWTH

Cardiac growth can be viewed as a system controlled by two sets of factors[36]: the intrinsic factors which are time-dependent, and more easily observed prior to the development of the circulatory system when hemodynamic forces are absent; and the extrinsic or function-dependent factors, which involve elements related to the contractile activity of the heart such as compensatory hypertrophy which follows an increase in hemodynamic load.

a - Intrinsic, time-dependent factors

Perhaps the best example of intrinsic regulation of growth is the cytodifferentiation of myocardiocytes[37,38]. The development of the heart begins with the proliferation of undifferentiated myogenic cells (premyoblasts). The first sign of overt myogenesis is the elongation of the premyoblasts. Some cells then initiate synthesis of muscle-specific proteins which culminate and eventually become cross-striated myofibrils in the developing myocytes. The differentiation process in the heart is not synchronized and consequently in the early developmental phase two cell populations are present at the same time: premyoblasts; and developing myogenic cells. Eventually all cells acquire myofibrils and no undifferentiated myogenic cells remain in the heart. As the myofibrillar mass accumulates the myocytes are gradually transformed into cylindrical and fully functioning adult myocytes. Mitotic divisions at this advanced stage still occur[39,40], although at a low rate.

All the stages of myogenesis are governed solely by intrinsic factors because the circulatory system has not yet developed. The time dependent, programmed character of early myogenesis is also evident from experiments demonstrating that primary cultures derived from embryonic hearts follow the same developmental pattern as cells within the intact embryo[36]. Further evidence comes from studies with skeletal muscle showing that cells in a clone derived from myogenic cells eventually express muscle characteristics[41].

The decline in mitotic activity observed for myocardiocytes is perhaps the best example of the general nature of time-dependent factors involved in growth regulation[36]. Although the regulation of mitotic activity in myocytes as well as in other cells remains obscure, several hypotheses exist. For example, it has been proposed that the gradually accumulating cell-specific structures within the myocytes present a hindrance to mitotic division of the nucleus[42]. Evidence supporting this hypothesis includes the observation of increasing cell cycle length with increasing myofibril accumulation; and the contradistinction of myocytes which contain a relatively small volume of myofibrils (amphibian ventricle, mammalian atria) and are able to resume DNA synthesis after injury, with the myocytes containing a larger proportion of myofibrils (mammalian ventricle) which never resume DNA synthesis after injury[36]. In addition, myofibrils of cells during mitotic activity become dispersed and gradually restored after mitosis[41]. Although this hypothesis is consistent with many observations there are also some exceptions that argue against its general applicability, such as the observation that neonatal rat myocardiocytes are capable of cytokinesis even while contracting without disruption of myofibrils[43].

A second hypothesis postulates that the loss of proliferative capacity is a consequence of a specified mitosis leading to a new phenotype cell: a mitosis of an undifferentiated myogenic cell abruptly produces a daughter cell which differs from its parent cell[44]. The daughter cell produced by such a critical mitosis is visualized as withdrawing from the cell cycle by repressing DNA synthesis and initiating synthesis of cell-specific proteins. This hypothesis obtained its support from studies of skeletal muscles in which synthesis of DNA and of cell specific proteins (e.g., myosin) do not occur at the same time[36].

In the heart, however, these two processes are not mutually exclusive[39,40], but it has been argued that possibly the heart requires more time for reprogramming, and consequently the repression of DNA may not be effective until one or two additional mitoses[36]. This argument has been challenged on the basis of the ability of developing myocytes to divide for several generations[45], along with evidence that adult myocytes can resume mitotic activity after a long silence as shown for morphologically dedifferentiated adult myocytes in long-term culture, and myocytes in injured portions of atria[36].

There is some evidence showing that genetic expression in eukaryotic cells may be regulated by specific DNA-associated proteins[46,47]. Jackowski et al[48] have demonstrated the differential appearance of three groups of non-histone nuclear proteins in Wistar rat myocardiocytes during postnatal development (4 days to 15 months of age). The time period when these proteins appear correlated with the cessation of the hyperplastic response of myocardiocytes to sustained stress. They have also shown that poly ADP-ribose polymerase activity is elevated in younger animals suggesting a role of poly ADP-ribosylation of specific non-histone chromosomal proteins as part of the complex metabolic signal that connects the bioenergetic response of sustained stress to gene activation[49].

In more general terms, several theories have been proposed concerning the mechanisms of cell division regulation which may be common for all differentiating cells. One theory cites the spatial arrangement of membrane components as the signal regulating cell division[50]. This is based on the demonstration that cell division can be influenced by alterations in the mobility of membrane components such as microtubules and microfilaments. Bundles of microfilaments have been identified under the membrane of normal cells that are undergoing characteristic changes in their shape while dividing or quiescent, this is in contrast to virally transformed cells which do not change shape and contain relatively smaller amounts of microfilaments[51].

A second theory proposes that the characteristic decline in proliferation capacity in a given cell is genetically programmed. For example, cultured human fibroblasts double only limited numbers of times before they lose their capacity to divide, deteriorate and eventually die[52]. The number of divisions in culture is related to the age of the donor and to the life span of its species[36].

It is questionable whether a genetically programmed number of cell divisions occurs in the heart. It has been shown that factors that affect total body growth also affect the number of cell divisions in cardiac myocardium. By adjusting the number of newborns per litter, thus manipulating the rate of body growth, it was observed that in smaller litters postnatal growth was increased and myocyte proliferation was enhanced as derived by histometrically determined cell number[53], and density of tritiated thymidine nuclear labeling[54]. Possibly the genetic apparatus is involved in the regulation of cytodifferentiation other than by determining the total number of divisions. For example, in a study comparing normal skeletal muscle myoblasts with myoblasts transformed with either viruses or chemical carcinogens, it was shown that the transformed cells remained in a proliferative phase and never initiated expression of muscle-specific characteristics[55]. In contrast, normal cells underwent a limited number of divisions leading to postmitotic myoblasts, once quiescent, myosin and other muscle specific proteins were synthesized and cells eventually contracted.

Genetic regulation is also suggested for cells traversing the restriction point from the G_1 to S phases of the cell cycle[56,57]. The activity of these genes depends on conditions such as cell size, nutrition, and growth factors.

b -The Homeo Box

A very recent and intriguing advance in the understanding of the genetic regulation of embryonic development comes from studies with Drosophila[58-61], and has been reviewed in more detail[62]. Studies on mutations have helped identify at least three classes of genes that control the spatial organization of the Drosophila embryo:

maternal-effect genes, which are expressed during oogenesis and specify the structure and the spatial coordinates of the egg; segmentation genes, which determine the number and polarity of the body segments; and homeotic genes, which specify segment identity. An example of an homeotic mutant is Antennapedia, in which the antennae of the fly are replaced by a pair of middle legs.

A potential relationship between the functions of homeotic genes has emerged with the discovery of sequence homologies between the homeotic loci of Drosophila pattern formation genes such as Ultrabithorax, Antennapedia and Fushi tarazy. A 180 base pair region located near the 3' end of the transcription unit of each of these loci is common to all of them. The homologies range between 75% and 79%. This sequence was called homeo box[58], since the cross homology is limited to a short and highly conserved DNA segment. The homeo box has been found in many other organisms including vertebrates and man. Using the Drosophila homeo boxes as probes, the first vertebrate homeo box was cloned from a Xenopus gene library and the sequencing data indicate a remarkable degree of sequence conservation[63]. Conceptual translation of the DNA into the corresponding protein sequence indicates that the conservation is higher at the protein level (75-87%). This protein domain, homeo domain, has a significant homology with the homeo domain and amino acid sequences encoded by the α 1 and α 2 genes which code for proteins controlling cell differentiation in yeast[64]. The high degree of conservation between the homeo box sequences from Drosophila[58-61], frogs[63], mice[65], and man[66] suggests the homeo box serves an important function that has been conserved during evolution. It is possible that the basic mechanisms of development may be more universal than previously thought.

c - Extrinsic, function-dependent factors

Adjustment of cardiac mass to hemodynamic load is a fundamental characteristic of the heart. The effect of contractile activity on the myocardium is difficult to study; unlike skeletal muscle, the heart is an autonomous organ whose function cannot be controlled as easily as the firing frequency of motoneurons. Nevertheless, there are numerous examples of the function-dependent nature of cardiac growth both when its hemodynamic load is increased or when it is decreased. The first case leads to hypertrophy; the second to atrophy or to the regression of previously acquired hypertrophy. Several theories exist which attempt to explain the growth-regulating signal, although none are fully compatible with all the aspects of cardiac growth. Theories of function-dependent growth regulation are the subject of a more detailed review by Zak[36], and will only be briefly described in this review.

A correlation between increased tension in the ventricular wall and increased metabolic activity in the heart has been well established. According to the ATP depletion theory, the increased rate of ATP utilization results in temporal depletion of energy stores. This depletion is then sensed by a process that determines the mass of cellular components, including both synthetic as well as degradative pathways. The basic postulate of this theory has received support based on the observation that cardiac compensatory growth is often accompanied by increased oxygen consumption and ATP hydrolysis. Therefore, a drop in the energy potential of the myocardial cell (e.g., the concentration ratio of ATP to its hydrolytic products, ADP and inorganic phosphate) must occur. The magnitude of this drop and, more importantly, its temporal correlation to the induced growth are not altogether clear[67].

The stretch theory of growth regulation has its origin in the classical observations of Feng[68], who demonstrated that passive stretching of isolated skeletal muscle increases its oxygen consumption and heat production. In analogy, an enhanced preload and afterload results in the stretching of myocardial cells; it is conceivable that this stretch stimulates metabolic pathways which, in turn, lead to compensatory growth as originally proposed by Eyster et al[69]. The most solid support for this theory comes from reports in which muscle enlargement was induced by various forms of passive stretching, such as studies with denervated diaphragm[70,71] and latissimus dorsi muscle[72], and when compensatory hypertrophy was induced by the elimination of a synergistic muscle either by extirpation, tenotomy, or denervation[73,74]. The mechanisms of growth-inducing effects of stretching were investigated only recently and consequently, very little is known.

The theory that hypoxia acts as a growth stimulus proposes that increased ventricular wall stress will result in oxygen deficiency in overloaded muscle cells, in turn the decreased partial tension of oxygen will derepress biosynthetic activities. Evidence supporting this theory comes from the observation that primary cultures of chicken embryonic heart cells incorporated progressively less radiolabeled precursors into proteins and nucleic acids as the partial pressure of oxygen in the gas phase was increased from 5% to 80%[75], and also from the fact that the growth of the heart decreases after birth when the pO_2 in arterial blood increases dramatically, relative to values measured in utero[36]. Further support comes from experiments with rats reared in an hypoxic environment up until 21 days of age, they had an enhanced rate of division of cardiocytes as shown by increased organ weight, DNA content, and incorporation of ^{3}H-TDR[76].

The common denominator for function-related cardiac growth appears to be the extent of the energy utilization during actin-myosin interaction[36], and possible links between the rate of energy utilization and the expression of specific genes have been suggested.

3 - HUMORAL FACTORS REGULATING GROWTH

Growth rate of cells in culture can be manipulated by plating density and cellular environment[77,78]. Humoral regulation of cell growth are quite numerous, although relatively little is known about the chemical nature and mechanism of the regulating factors involved[36]. Among the various agents involved in growth regulatory processes, one group has become particularly prominent in the last several years. Collectively referred to as polypeptide growth factors (PGF's), these molecules have emerged as a major subset of hormones that, in terms of total numbers, will probably far eclipse the more classical endocrine polypeptides. Several PGF's have been partially purified, isolated to homogeneity, or fully structured. Although no single description or definition encompasses all PGF's it has been proposed that these substances meet the following requirements[79]: (1) the majority of the structure consists of polypeptide material; (2) initiation of response occurs external to the target cell; (3) responses are initiated exclusively by the formation of specific receptor complexes; (4) a specific hypertrophic or hyperplastic response results from the formation of the hormone receptor complex; and (5) the PGF and its receptor are removed from the cell surface by receptor-mediated endocytosis for the minimal purpose of degradation (or receptor recycling).

The PGF's interact initially with membrane-bound receptors that protrude from the external side of the cell surface. These are largely, if not exclusively, intrinsic membrane glycoproteins that can be solubilized in nonionic detergents without significant loss of ligand-binding affinity and, not unexpectedly, show both high affinity and tight specificity for their ligands. The molecular weight and subunit structures of the PGF receptors, with the exception of those for insulin and IGF-I, do not show obvious relationships despite their involvement in growth regulation. This may reflect the independent evolution of each ligand-receptor pair, despite the clear ancestral relationship of some of the hormones themselves[80,81], or the obscuring of the structural relatedness that may be evident from further sequence studies.

The identification of the tyrosine-specific protein kinase activity of several PGF receptors comes from the incisive observations of Cohen et al[82-84] with EGF and the human epidermoid cell line A431. Characterization of the EGF-sensitive phosphorylated products, including the receptor, revealed the surprising result that the amino acid modified was tyrosine[85]. This site of phosphorylation is relatively uncommon, as compared to serine and threonine, the major residues modified by cyclic nucleotide or calcium-dependent protein kinases. The finding that the EGF receptor apparently contains as an integral part of its structure, a tyrosine-specific protein kinase capable of phosphorylating itself and a variety of other substrates was followed by similar reports for insulin[86-88] and

PDGF[89,90]. Of particular interest is the fact that tyrosine-specific kinases are also encoded by several tumor viruses[91] such as Rous sarcoma virus[92-94] and Abelson murine leukemia virus[95]. The Rous sarcoma virus protein has a molecular weight of 60,000 and contains covalently attached phosphate. The cellular cognate, coded by the cellular oncogene c-src, is also a tyrosine-specific kinase of similar properties. As with its viral counterpart, it has been located near adhesion plaques[91], which are specialized areas of the plasma membrane associated with substratum contacts. Its function in normal cells is unknown although it may be related to the growth response.

Despite the considerable advancement in the understanding of the structure of polypeptide growth factors and their receptors, no clear-cut mechanism of action for these substances has been deduced. However, certain general characteristics common to the activity of all PGF's seem evident[79]: (1) initiation of the response by formation of a specific, high-affinity cell surface receptor complex; (2) generation of an immediate signal that is responsible for at least a part of the overall mechanism; (3) internalization of both the ligand and its receptor largely, if not universally, through the mediation of clathrin-coated pits; and (4) the ultimate degradation of both the receptor and ligand by the action of lysosomal enzymes.

It should be noted that the specific response for all

PGF's is not the same and neither is the mechanism of action likely to be. As an example, the PGF's stimulating mitogenic responses have been subdivided into two groups termed progressive and competence[96]. Factors in the progressive category are able to stimulate cells to pass into S phase in the absence of additional agents, whereas competence factors are only able to prime the cells but require assistance in the form of additional factors to bring on the onset of DNA synthesis. However, other workers have noted that competence factors may only require proper tissue culture conditions to act in a progressive manner[97].

The interaction of PGF's with plasma membranes generally produces a trophic stimulation that along with other responses is characterized by metabolic uptake and modulation of ion fluxes[98]. These changes presumably reflect activation of appropriate transporter molecules in the membrane, rather than simply changes in membrane porosity. The net effect is to cause an increase in the anabolic metabolism that may serve to prepare the cell for the replication process[99]. However, an entirely similar response is found in peripheral neurons stimulated by NGF[100], which do not undergo cell division, indicating the more general nature of this pleiotypic activation[101].

Among the rapid effects stimulated by PGF's, phosphorylation of various intracellular proteins has received considerable attention and findings further reveal the complexity of the PGF's mechanism of action. In sympathetic neurons, for example, nerve growth factor (NFG) causes the increase in phosphorylation of two proteins of molecular weight 30,000, one which migrates with histone H1 but is distinct from it[102]. In the pheochromocytoma cell line PC12, however, NGF and also EGF, phosphorylate only one of these proteins. A more detailed analysis of NGF-induced phosphorylation in PC12 cells revealed that tyrosine hydroxylase, ribosomal protein S6, histone H1 and H3, and HMG 17, a nonhistone chromosomal protein, among others, were increased while histone H2A was actually reduced in extent of modification[103]. Cholera toxin and cAMP produced a similar pattern, and EGF and insulin phosphorylated a different subgroup. The change in ribosomal protein S6 was additive for saturating levels of NGF, EGF, and insulin, suggesting different pathways for each hormone. It has been suggested that cAMP is not involved[98,100,104]. A decrease in phosphorylation of another soluble protein of molecular weight 100,000 in NGF-treated PC12 cells was recently detected[105]; the effect was also observed in cell-free extracts[106]. In addition, PGF's have been shown to have varying patterns of phosphorylation in studies with an established line of G_0-arrested Chinese hamster lung fibroblasts. Fetal calf serum was observed to induce the rapid phosphorylation of three proteins, a nuclear 62 kd protein, a cytoplasmic 27 kd protein and ribosomal protein S6[107]. Eye derived growth factor (EDGF) and PDGF caused the phosphorylation of the 27 kd protein at low concentrations and all three proteins at high concentration. In contrast, insulin and IGF-II only stimulated the phosphorylation of S6, providing further evidence, at the molecular level, for the division of mitogenic PGF's into two groups[96].

4 - CARDIAC HYPERTROPHY IN NEWBORN SHR

Cardiac hypertrophy in SHR is observable at birth[1,108-113]. The significance of cardiac hypertrophy associated with a genetic predisposition for hypertension is a controversial issue. Cutilletta et al[108] have reported higher right and left ventricular weight and more DNA per gram of tissue in newborn and two week-old SHR. Left ventricular hypertrophy was found in SHR from 1 day to 24 weeks old, and right ventricular hypertrophy was also noted in SHR from 1 day to 8 weeks old. From these results they suggested that the higher DNA content may reflect hyperplasia in newborn SHR heart, and that the appearance of cardiac hypertrophy so early in the hypertensive process may reflect an intrinsic abnormality in SHR heart, not caused by pressure overload. If a primary abnormality was present in an animal genetically predisposed to hypertension, they proposed[108,114] that this could result in myocardial dysfunction and hypertrophy. The scheme that would follow might be a compensatory increase in sympathetic activity to maintain cardiac output returning myocardial function towards, but not completely to normal. Vasoconstriction resulting from the increased sympathetic activity would then produce an increase in peripheral vascular resistance and an elevated systemic pressure. They also suggested that other genetically determined factors, such as increased reactivity of the arteriolar musculature[115] or increased levels of circulating catecholamines[116], may play a role. As a result of the high peripheral vascular resistance and continued hypertension, cardiac output would be further diminished and the left ventricular hypertrophy would increase.

A primary myocardial abnormality in newborn SHR was also suggested by Hallback-Nordlander[109]. In a study comparing left and right ventricular weights in newborn, 6, 15, and 28 week old Wistar (WAR), WKY, and SHR rats; left and right ventricles were significantly heavier in all SHR rats than in age matched controls. However, the left/right ventricular weight ratio was increased only in 15, and 28 week old SHR and not at birth and 6 weeks. It was suggested that if the cardiac hypertrophy in SHR at 6 weeks was predominantly the result of an increased pressure, then the left/right ventricular ratio should already be increased, since the left ventricle after birth would alone have to cope with an increased systemic pressure. Therefore, the structure of the cardiovascular system in SHR must already be somewhat altered at birth possibly due to hormonal trophic influences.

In our laboratory[1,110] a study was designed to establish whether neonatal hyperplasia was confined to the heart or was a generalized phenomena. Relative organ weight (mg/g body weight), protein, and DNA content (mg/mg organ weight), of newborn heart, kidney, and liver from WKY, SHR, and WAR were compared. The results confirmed not only the presence of cardiac hyperplasia, but also established the presence of renal hyperplasia in SHR. These properties were present despite lower body weight and lower relative weight, protein and DNA content for SHR liver. Four hypotheses were formulated to explain these observations. First, cardiac and renal hyperplasia could be a response to hemodynamic growth stimuli which are present in hypertensive SHR mothers and which act on their fetuses during prenatal development altering the growth of these two organs. This hypothesis is not strongly supported since information on the relationship between maternal and fetal blood pressure in rat is not available. Secondly, the heart and kidney but not the liver in SHR may possess a specific genetic "code" for more rapid growth. Abnormal growth has been reported for SHR derived aortic smooth muscle cells[117,118]. Thirdly, it is possible that SHR may carry some circulating growth factor(s) which could potentiate the growth of specific organs, such as the heart and kidney. This possibility is compatible with a recent study[119] showing that vascular smooth muscle hyperplasia can be induced in veins of WKY by cross-circulating WKY with SHR. Additional support for altered blood factors in SHR was shown by the development of sustained increased blood pressure in genetically normotensive Sprague Dawley newborns nursed by SHR foster mothers[120]. Additional proteins have been observed in plasma from essential hypertensive patients and from SHR[121], although no evidence has been provided relating this abnormality to a growth defect. Fourthly, a different timing in the development of catecholaminergic fibers of the cardiovascular system may lead to the observed hyperplasia, since proliferation of myocardiocytes is temporally related to the maturation of these fibers. This idea is in accordance with the observation that the number of catecholaminergic fibers in jejunal arteries of SHR is significantly higher than WKY at 2 weeks of age[122].

With the discovery of cardiac and renal hyperplasia in newborn SHR, it was of interest to see if this abnormality was common to other rat models of hypertension. Our laboratory, in an international study, compared newborns from models of essential hypertension (Kyoto, Montreal-NIH, Dunedin and Lyon strains), renal hypertension (Milan strain) and experimental hypertension (DOCA-salt treated Wistar rats-PRAHA) with their appropriate normotensive controls[123]. The hearts, kidneys, and livers of these newborns were collected on site at the various collaborating research centers and sent to our laboratory for protein and DNA determinations. From this study three distinct patterns of relative protein and DNA contents were revealed (**Figures** 1 and 2). The first, cardiac and renal hyperplasia (usually accompanied by decreased liver relative protein and DNA contents), is shared by the Kyoto, Montreal-NIH, Dunedin, and Lyon strains of genetically-hypertensive rats, which are examples of spontaneous hypertension. Secondly, a decrease in relative protein and DNA contents for heart, kidney, and liver was observed for the renal hypertensive Milan strain; and thirdly, the offspring of the mineralocorticoid (DOCA-salt) hypertensives had increased relative protein and DNA in all three organs studied, perhaps an indication of compensatory anabolic activity in the fetuses. The similar patterns of relative protein and DNA contents within the group of essential hypertensive models which develop hypertension spontaneously, supports the concept that a causal relationship may exist between cardiac and renal hyperplasia and hypertension. It is important to note that if maternal blood pressure was the only factor producing the pattern of alterations in the essential hypertensive model group, one would expect a similar pattern of organ protein and DNA content for the offspring of the DOCA-salt induced hypertensives.

Gray[113] also reported significantly increased relative heart weight (mg/g body weight) both in newborn and 10 day-old SHR as calculated on both a wet and dry weight basis. Gray[109] and Bruno et al[124] provided evidence of

Figure 1

Summary of organ protein content/body weight differences (percent of normotensive controls) in newborn rats from essential, renal, and experimental models of high blood pressure (HBP). Essential hypertensive models included: stroke-resistant (SR) and stroke-prone (SP) strains of the spontaneously hypertensive rat (SHR), SHR Montreal-NIH, Dunedin, and Lyon strains. The Milan strain is a model of renal hypertension, and the experimental model was the DOCA-salt treated Wistar rat-PRAHA. This figure was adapted from Pang et al[123].

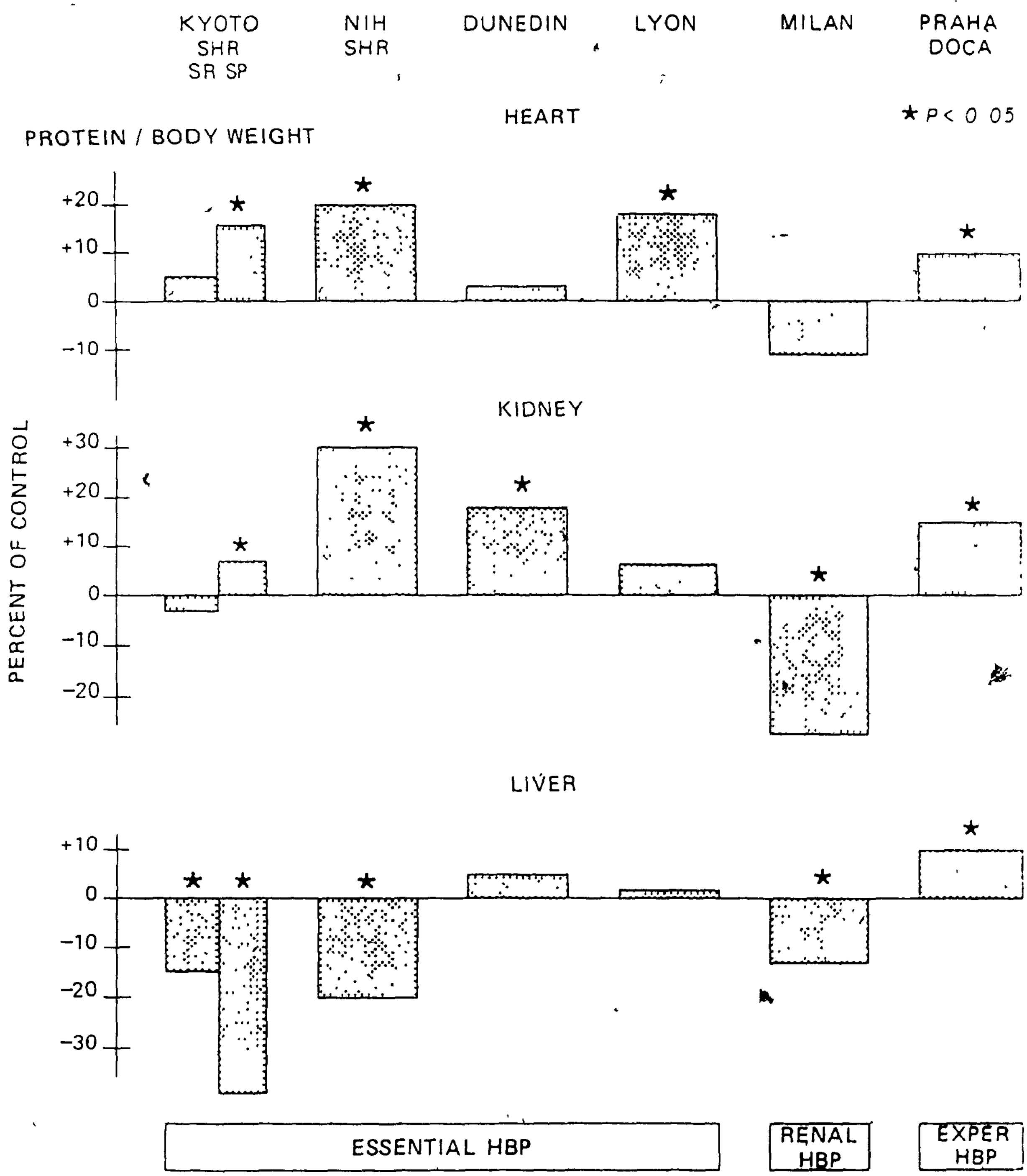
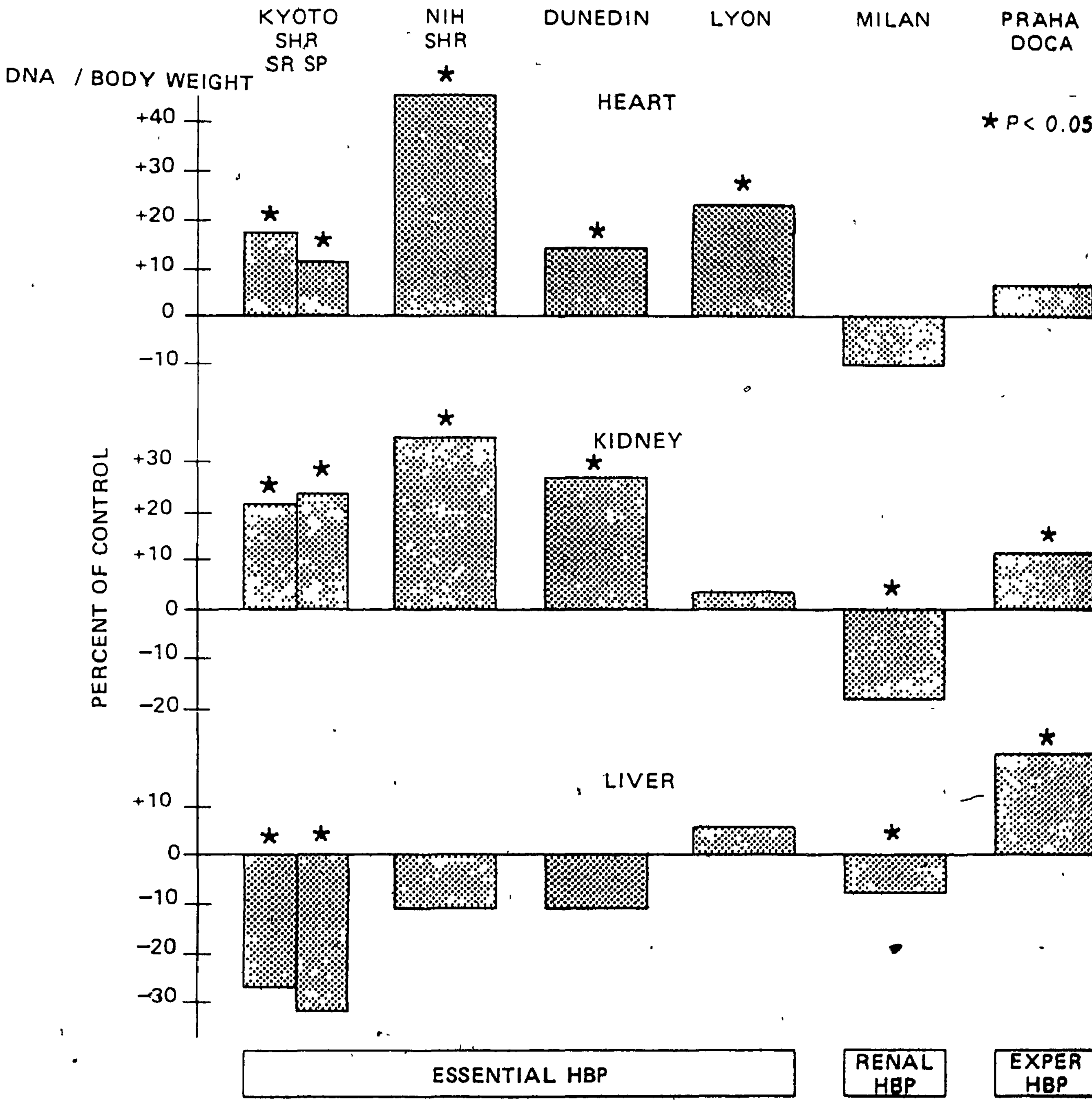

KYOTO
SHR
SR SP
NIH
SHR
DUNEDIN
LYON
MILAN
PRAHA
DOCA
HEART
★ P< 0 05
PROTEIN / BODY WEIGHT
+20
+10
0
−10
KIDNEY
PERCENT OF CONTROL
+30
+20
+10
0
−10
−20
LIVER
+10
0
−10
−20
−30
ESSENTIAL HBP
RENAL
HBP
EXPER
HBP

Figure 2

Summary of organ DNA content/body weight differences (percent of normotensive controls) in newborn rats from essential, renal, and experimental models of high blood pressure[1 2 3].


KYOTO SHR SR SP
NIH SHR
DUNEDIN
LYON
MILAN
PRAHA DOCA
DNA / BODY WEIGHT
HEART
KIDNEY
LIVER
* P< 0.05
PERCENT OF CONTROL
+40
+30
+20
+10
0
-10
-20
-30
ESSENTIAL HBP
RENAL HBP
EXPER HBP

elevated mean arterial blood pressure in newborn SHR and caution against disregarding elevated blood pressure as a contributing factor to the cardiac hypertrophy in SHR at birth.

5 -THE HEART IN HYPERTENSION

In summary, it is by no means an established fact that the hypertrophy in SHR is due only to stress induced by an increased afterload; the reports that hypertrophy exists at birth and probably in utero may indicate a genetic predetermination for increased ventricular and renal mass. Other indications that this interpretation has validity are presented by the studies involving attempts to prevent or reverse the hypertrophy by pharmacological intervention at the fetal or neonatal stage. Reversal of the hypertrophy is less likely with antihypertensive agents[125-131] than is the reduction in pressure or heart rate. Since a pressure rise is occurring simultaneously with the increase in cardiac mass, as Gray[132], so cogently stated, it may be difficult to determine how the two factors are associated.

Whether the heart can initiate some forms of hypertension has been the subject of much debate[133,134]. The concept of "cardiogenic hypertension" or hypertension in which the heart plays a pivotal role in the pathogenesis of the disease[135], has gained support on two basis[133]. The first of which is that an increased cardiac output, although not common in the established phase of the majority of hypertensions, was frequently found in the early phases of both essential and secondary hypertensions[136-138]. The second bases is the importance of pressor reflexes originating from the heart, coronary arteries, or the aorta, which may provoke severe hypertension with an increase in peripheral resistance[139]. Thus characterization of hypertension by a high systemic resistance does not necessarily rule out a possible cardiac dysfunction[133].

Left ventricular hypertrophy is a major complication of hypertension. The factors dictating the evolution of left ventricular function in the presence of hypertrophy are numerous. Long standing hypertrophy, however, usually is associated with reduction of systolic function caused by the hypertrophy itself, possibly due to the outstripping of its own blood supply as well as diminished neural response[140]. There is some evidence that left ventricular hypertrophy associated with hypertension is reversible[141-143], and studies with SHR[144] and humans[145-147] suggest benefits of treatment in preventing and reversing cardiac hypertrophy.

D - QUANTITATIVE CELL CYCLE ANALYSIS

Cytokinetics, the analysis of the cell cycle behavior of cell populations has gone through three dramatic phases in the past 30 years. The first began in 1953 with the demonstration that DNA was synthesized in interphase[148] and, hence, that the cell cycle could be divided into four phases. The second began with the introduction of tritiated thymidine[149,150] in 1957 and its extension to detailed kinetic analysis[151]. The third major phase began with the development of flow cytometry[152-154] and sorting[155,156].

1 - THE CELL CYCLE

a -Overview

The cell cycle is extremely complex and our understanding of it is very limited. Boynton and Whitfield[157] provide an excellent, extensive review of cAMP and cell proliferation. The cell cycle of most, but not all eukaryotic cells can be divided into four phases[157-160]. It begins with the birth of the cell in phase M, mitosis-cytokinesis, as the cell ages it goes through a G_1 (or Gap 1) phase, proceeds through an S (DNA-synthetic) phase, during which it replicates its chromosomes; this is followed by a second gap, or G_2 phase, and finally ends its life as an individual upon dividing into two new individuals.

Variation in cell cycle times among different cell types is mainly due to variation in the length of G_1, with the duration of S (6-8 hrs), G_2 (3-4 hrs), and M (1 hr) being relatively constant for cells in culture[156]. In addition, there is much variability in the length of G_1 among individual cells in a single population. Several models have been proposed to explain this variability.

According to the deterministic model[161], the G_1 phase variability is due to phenotypic differences in cells at birth. Therefore, newborn sister cells, despite their common origin, will need different lengths of time to reach the critical size and composition required for DNA synthesis initiation. Cells must enter S with a relatively uniform composition, therefore, the lengths of S, G_2, and M are less variable that G_1.

In the simple random transition model[162] the cell cycle is divided into a probabilistic A state and a deterministic B state. The B state includes S, G_2, division phases, and an early part of the succeeding G_1 of the daughter cells. At its birth the cell is still in the B state of its parent, finishes a programmed sequence of postmitotic events and then enters the A state. The A state is sort of a limbo into which the young cell remains until the randomly fluctuating level of one or more scarce components exceeds the level necessary for entry into S phase. Thus the length of the cell cycle is a function of the determined duration of chain of events in the B state and the probability of the random transition from the A to B state. The simple random transition model does not account for the variability within and between sister cell pairs, and also does not explain the delay in DNA synthesis following serum addition to cells rendered quiescent by serum deprivation[157,163,164]. To compensate for these deficiencies the complex random transition model was devised[164]. This model contains two non-deterministic, growth factor modifiable states, A and Q, and a deterministic L state. The serum deprived cell may still randomly leave the A state and enter the Q state, an event that includes DNA synthesis initiation. However, this cell and its daughters will remain in the Q state until serum is added to the medium. With serum addition the probability of the Q to L transition increases and the quiescent daughter ~~cells~~ enter the long deterministic sequence of events in the L state, which ends in the A state. If the medium remains adequate the probability of the Q to L transition will approach 1.0 and the cycle is reduced to the simple random transition model with the random exist from the A state leading to DNA synthesis initiation and the L state. Thus two pairs of control points exist, A to Q, which triggers DNA synthesis, and Q to L, which triggers a series of growth events.

A noncycling cell according to the complex random transition model is simply in a Q state with a low probability of randomly entering the L state. The more flexible deterministic model would allow such nonproliferating cells to be blocked indefinitely in the G_1 phase, or to be in a proliferatively inactive state with repressed cell cycle genes[157]. It is likely that the ultimately successful model will contain elements from both current models[157].

b - THE G_1 PHASE

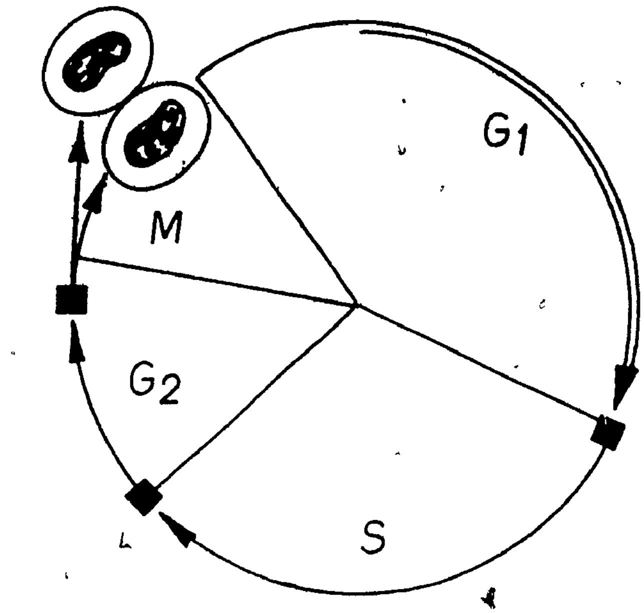
The events responsible for the initiation of S phase occur during G_1. Protein synthesis is needed, but the exact nature of the events is unclear. The G_1 phase of the cell cycle is the most responsive to growth factors and hormones and is the most sensitive to inhibition by shortages of essential nutrients, because conditions must be perfect for the cell before risking the initiation of DNA synthesis[157,159,161,165,166]. Presumably this is a safequard against injury and death that would occur following premature arrest in the S phase[157,165].

Boynton and Whitfield[157] describe four main types of prereplicative phases, types 1 and 2 will be briefly discussed here (**Figure 3**). The type 1 prereplicative phase is described as the basic or minimum sequence of G_1 events that starts immediately after telophase and continues uninterrupted to the initiation of DNA synthesis[157]. For example, cells synchronized by mitotic selection or "shake-off" are in type 1 phase.

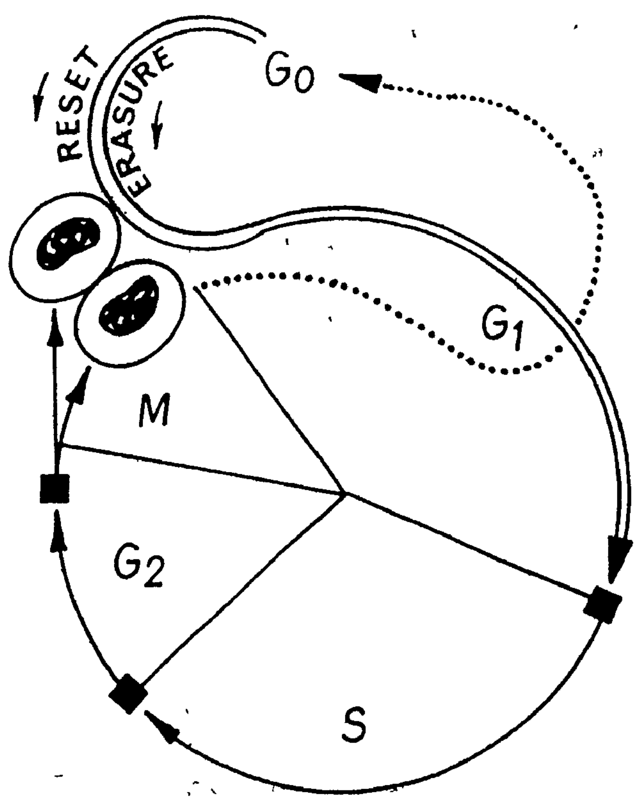
The second type of prereplicative phase follows stimulation of quiescent cells exposed to suboptimal growth conditions [157]. Under these conditions the cell will stop cycling during the G_1 phase in order to avoid danger of premature arrest in S phase. The cell will stop cycling at a point appropriate to the missing component called the execution or restriction point[161]. The cells will bear the marks (e.g., less condensable chromatin[167],

Figure 3

The prereplicative phases of eukaryotic cells. Type 1 is the basic or minimum prereplicative phase of continuously cycling cells of established lines. Type 2 is the prereplicative phase following the stimulation of quiescent G_0 cells that requires a preliminary resetting of the cell to an early posttelophase state by erasure of such vestiges of a previous aborted transit through the G_1 phase (dotted line). This figure was adapted from Boynton et al[157].



TYPE 1



TYPE 2

monophosphorylated histone H1[165], a high cyclic AMP content[168], and ciliation of its centrioles[169] of the stage of prereplicative development it reached before stopping[157]. The cell will then lapse into a reversible G_0 state[159,170,171]. The existence of the G_0 state as a qualitatively distinct state receives support from observed kinetic and biochemical differences between G_0 and G_1[161], and from the observation that G_0 cells take longer to reach S than do G_1 cells progressing directly to S from mitosis[172]. Conditions which shift cells into G_0 include: high cell density[173]; serum limitation[174-177]; limitation of some amino acids[174,178,179] or of other nutrients, such as phosphate, glucose[178,179], or lipids and biotin[180]; and the presence of certain drugs[181,182]. There is some controversy as to whether cells arrested in G_0 by different mechanisms are actually in identical physiological states[181,183,184,185].

Inactivation of the pivotal polypeptide chain elongation initiation factor (eIF-2), is one way, if not the main way, the G_1 cell shuts itself down in response to suboptimal growth conditions[157]. This event stops all protein synthesis[186], which is followed by reduced transport of small precursors, disaggregation of polysomes, accumulation of 80s ribosomes, reduced ribosomal RNA synthesis, enhanced protein degradation, and inhibition of DNA synthesis[157,165,186,187]. The longer a cell stays in the G_0 state, the longer it will take to initiate DNA synthesis when stimulated to resume cycling[157,188]. The type 2 G_1 phase following this stimulation is different from the type 1 G_1 phase, because the cell starts out with vestiges of its previous abortive transit through the G_1 phase, and must first erase and reset itself to a late teleophase-like state[157]. Therefore, the monophosphorylated histone H1 is dephosphyorylated, the centrioles are deciliated, and the cyclic AMP drops during the erasure and reset[157,165,189,190].

c - The S Phase

The S phase is not just a period of DNA synthesis, because the chromosomes are also composed of two major classes of proteins, histones and nonhistones. Chromosomes are very long coiled and supercoiled strings of nucleosomes, each of which is an octamer of histones or histone cluster (two molecules each of histones H2A, H2B, H3, and H4) around which is wound the duplex DNA strand, forming a nucleohistone[160,191]. The remaining histone H1 is not part of the histone cluster, but links DNA and the nucleosome core histones, and somehow controls chromosome coiling or condensation[157,160,191]. The interaction between DNA and histone is primarily an interaction between DNA's acidic phosphates and histone's hydrophilic basic amino acids, argenine and lysine. The genes coding for the histones are activated at the beginning of the S phase, and the synthesis of these proteins and their assembly into nucleosomes are tightly coupled to DNA synthesis[191]. The relatively long DNA strands of the several chromosomes in a typical eukaryotic cell have multiple initiation sites for its replication. This enables the chromosome to be replicated and the cell to divide in a reasonable short time[157,160,161,192]; for example, in human cells, average DNA replicative rate is about 0.5μ/min. In a mammalian cell each of the approximately 30,000 initiation sites is in the middle of a 15 to 60μm replication unit (or replicon), the two replicating forks (hence two DNA-synthetic enzyme complexes) move bidirectionally outward from this site[157,160,161,192]. Not all the initiation sites are activated during an S phase and the number of these sites (hence the average length of the replicating units and the rate of DNA synthesis) varies according to cell type and developmental stage[157,160,161,192]. In eukaryotic cells, the actual event that triggers initiation at an origin of replication is unknown. An early event could be the generation of a nick, providing ends for DNA or RNA polymerase[160].

The replication units are organized into clusters of 100 adjacent units, which initiate DNA synthesis synchronously under the control of a leader region[160,192]. The replicating unit clusters are, in turn, grouped into a series of sequentially initiated banks or batteries, the components of which may be located on different chromosomes[161]. With the onset of DNA replication, the histone octamer cores of the old nucleosomes stay with the leading strand and the lagging strand is somehow unwound, or lifted, from the old nucleosome cores and stabilized by a DNA-binding protein. Once the lagging strand has been replicated, new histones bind to the DNA, with H3 and H4 being first and H2A and H2B next, to form new nucleosome cores[160]. The extra core H1 will bind much later[160]. The DNA synthesis initiation and subsequent DNA chain elongation in a cluster of replication units requires attachment to sites on the nuclear protein matrix where a local decondensation of the chromatin can occur and the DNA synthetic enzyme complexes can operate.

d -<u>The G_2 and M phases</u>

Once the chromosomes have been replicated the cells enter the G_2 phase. At this time the cell activates genes that code for the components needed for chromosome condensation, nuclear membrane breakdown, assembly of the mitotic spindle and cytokinesis[193,194]. When these newly synthesized factors reach a critical level, they trigger chromosome condensation and entry into mitosis.

Mitosis is an essential adjunct to chromosome duplication since it ensures that each daughter cell gets one sister of each chromatid pair and therefore a complete set of chromosomes[160]. The four mitotic stages, prophase, metaphase, anaphase, and telophase, are in fact arbitrarily defined, since mitosis takes place as a continuous process.

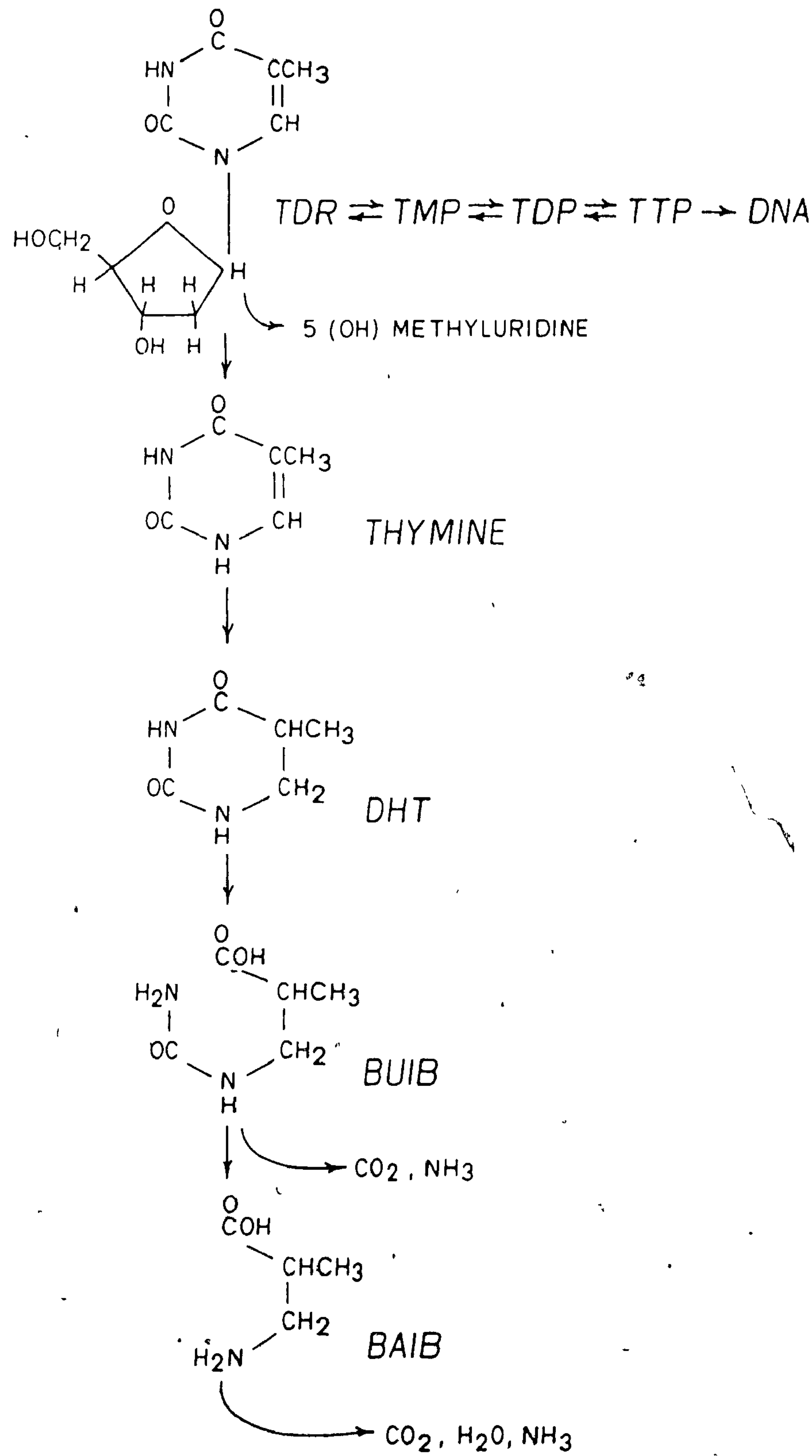
Mitotic prophase is heralded by the onset of chromosome coiling. As coiling and condensation progress, the two sister chromatids of each chromosome can often be distinguished, and the nucleolus becomes undetectable with the light microscope. The mechanism responsible for prophase chromosome condensation is unknown. The onset of metaphase is marked in most cells by the breakdown of the nuclear envelope, releasing the chromosomes and nucleoplasm to fill the greater part of the cell. The presence of a complete spindle is a characteristic of metaphase. The site of spindle microtubule insertion in the centromere region is called the kinetochore. With the chromosomal microtubule insertion the sister chromatid pairs become aligned in a plane through the cell midline. Mitotic anaphase begins with the division of the centromeres or more precisely, of the kinetochores. The sister chromatids of a given pair now separate, and each moves kinetochore first toward the spindle pole nearest to it. Once the chromatids have separated they are considered as daughter chromosomes. Once pole migration is complete, a nuclear envelope forms around each set of daughter chromosomes, a nucleolus reforms, the spindle microtubules disappear, the chromosomes uncoil, and each daughter nucleus gradually assumes an interphase morphology. These events characterize telophase, the final mitotic stage under most circumstances. Telophase is followed by cell division, or cytokinesis.

2 -TRITIATED THYMIDINE

The onset of DNA synthesis, S phase, can be measured with a pulse label of H^3-TDR which is only incorporated into newly synthesized DNA. Tritiated thymidine has been used extensively for autoradiographic studies. Thymidine (TDR) itself does not occur naturally on the main intracellular pathways that lead to DNA synthesis but is introduced into them by a single phosphorylation step to thymidine monophosphate, TMP (**Figure 4**). Although TDR is nonessential for DNA synthesis except in organisms which cannot make their own TMP, it is incorporated rapidly into DNA in most organisms. Thymine, in contrast, is incorporated much less efficiently than TDR, and its rate of incorporation into DNA in most mammalian tissues is less than 4% of the rate of TDR incorporation. The incorporation of TDR into DNA proceeds by a sequence of phosphorylation steps through TMP, TDP, and TTP followed by the assembly of TTP, together with the other nucleoside triphosphates, into DNA[195]. The enzymes involved in the phosphorylation steps are known as thymidine kinase, thymidine monophosphate kinase and thymidine diphosphate kinase, respectively, the latter two enzymes are commonly discussed under the combined term of thymidylate kinase. The reaction steps involving TDR and TDP kinases are relatively rapid compared to TMP kinase.

Figure 4

Thymidine (TDR) pathways of incorporation into DNA and degradation. The incorporation of TDR into DNA proceeds by a sequence of phosphorylation steps through thymidine monophosphate (TMP), thymidine diphosphate (TDP), and thymidine triphosphate (TTP). The major pathway of TDR degradation is through thymine, dihydrothymine (DHT), β-ureidoisobutyric acid (BUIB), β-aminoisobutyric acid (BAIB), to CO_2, H_2O, and NH_3.


TDR ⇄ TMP ⇄ TDP ⇄ TTP → DNA
5 (OH) METHYLURIDINE
THYMINE
DHT
BUIB
CO_2, NH_3
BAIB
CO_2, H_2O, NH_3

Thymidine kinase activity shows a high degree of correlation with cellular proliferation rate and DNA synthetic activity. This correlation has been observed in several transplanted tumors[196,197], in mammalian intestine[198], skeletal muscle[199], cardiac muscle[195], and retinal epithelium[200]. The incorporation pathway has also been found to have a high activity in rapidly proliferating tissues[196,201,202].

Of the three phosphorylating enzymes involved in the incorporation of TDR into DNA, only TDR kinase has been studied in detail up to the present time. Some progress has been made in isolation and characterisation of TMP and TDP kinases but these are more unstable than TDR kinase when extracted from the cell. TDR kinase itself is unstable in solution and decays with a half life of about 30 min at 38°C, but each of the enzymes can be stabilized by the addition of its respective substrate. TDR kinase has the same substrate specificity irrespective of the tissue of origin; deoxyuridine and 5-substituted derivatives including TDR and the halogenated derivatives are all substrates for the enzyme, but other deoxynucleosides or ribonucleosides are not phosphorylated to any significant extent. The TDR kinase reaction has an absolute requirement for ATP and Mg^{2+} ions in mmolar concentrations. Other divalent ions such as Ca^{2+}, Co^{2+}, or Mn^{2+} are much less effective in supporting enzymatic activity. The role of ATP in the reaction may be not only as a phosphate donor but also an activator, so that two molecules of ATP are required for each molecule of TDR and TDR kinase[203]. The molecular weight of TDR kinase is greater than 100kDa[204,205], and the enzyme exists in a number of different states of aggregation. The enzyme from mammalian cells, for example, occurs in an aggregated form in dilute solution and dissociates into six subunits when the ionic strength of the solution is increased[206]. TDR kinase consists of several subunits with three or four sites for attachment of the substrate (TDR), inhibitor (TTP), phosphate donor (ATP) and the activator (ATP) molecules, respectively. The end product of a sequence of enzymatic reactions is often found to regulate its own synthesis by means of end product inhibition (e.g., negative feedback)[202]. A particular example of this is the end product inhibition exerted by TTP on a number of enzymes. The kinetics of inhibition of TDR kinase by TTP may differ in different organisms[207]. Although TDR itself is not on the main pathways of intracellular DNA synthesis, TDR kinase is absent only from selected mutants of cells and viruses and from very few other organisms[208]. Other organisms may exist which do not incorporate TDR into DNA, however, this property is exceptional and TDR kinase is a widespread and common enzyme.

The first step in degradation of TDR consists of the cleavage of the glycosidic bond in TDR to form thymine and deoxyribose-1-P by TDR phosphorylase[209]. The phosphorylase is extremely widespread and is found in many mammalian tissues, plants and microorganisms. In mammalian tissues the phosphorylase is found, in the order of decreasing activity, in the intestinal mucosa, liver, bone marrow, kidney spleen, lung, and heart, and much less activity is found in the brain and muscle[209]. The major pathway of thymidine degradation is through thymine, dihydrothymine, β-ureidoisobutyric acid, β-aminoisobutyric acid, to carbon dioxide and water[210-212]. The final degradation steps to β-aminoisobutyric acid and CO_2, may only occur in the liver, spleen and kidneys, whereas the earlier steps, particularly the one involving the phosphorylase occur in many tissues[209].

Since TDR itself is not normally found on the pathways that lead to DNA synthesis inside the cell, the existence of TDR kinase has been an enigma. It has been difficult to find a reason for the existence of such an apparently superfluous enzyme. The problem, however, should not be viewed solely with reference to this one kinase. Although none of the deoxynucleosides occur naturally within the cell on the pathways of DNA synthesis, each are readily incorporated into the cell and phosphorylated by the appropriate kinase. These kinases are probably all salvage enzymes which are important for making use of the breakdown products from dead cells in the whole animal. Since most cells also contain 5'-nucleotidases and phosphorylases there is probably competition for the available TDR, or other nucleosides, between the synthetic and degradative pathways; the former pathway will then predominate when cells require TTP for DNA synthesis. Four main types of salvage process have been suggested[194,213] and may have different relative importance in different tissues. The four types of salvage are: (1) DNA from dead cells is incorporated by cells within the same tissue. (2) DNA from dead cells, having been broken down into relatively small molecules, is incorporated by cells throughout the whole organism. (3) The special case of the gastrointestinal tract where DNA from cells which are sloughed off into the lumen are absorbed lower down the tract and incorporated into epithelial cells. (4) The special case of the bone marrow, in which the nucleus is discarded at the orthochromatic stage in erythropoiesis or when there is an overproduction of cells and consequent cell death during myelopoiesis.

E - HEAT STRESS

1 - INITIAL STUDIES

In 1962, Ritossa et al[214] reported the induction of specific puffs on Drosophila chromosomes after a brief treatment of larvae at supraoptimal temperatures. In addition, they showed that these newly induced puffs were involved in RNA synthesis[215]. In view of the speculation that puffs represent sites of active genes[216], it was shown that heat shock proteins (hsp's) are actively synthesized following heat shock induction[217,218], as a result of the translation of specific messengers transcribed at the heat shock puff sites[219-221]. This response to heat has subsequently been studied mainly in Drosophila as a model system for investigating the regulation of gene activity. However, in recent years similar responses to heat have been observed in other organisms. This ubiquitous responsiveness of cells, from bacteria to man, points to the fundamental importance of the heat shock response in living cells. In Drosophila several of the heat shock genes have been sequenced[222-226]. Thus while the structure of heat shock genes is well documented, there is still very little information on the mechanism of the cellular response and the function of the hsp's[227]. Several types of hsps can usually be observed : hsp 70, the most conserved and ubiquitous hsp; hsp 83, synthesized at low concentrations under normal conditions; and a group of small proteins (hsp 22-30) encoded by genes related to one another.

2 - GENETIC REGULATION DURING HEAT STRESS

Early studies in Drosophila have shown that both transcriptional and translational regulation mechanisms operate during heat shock. The rapid activation of the heat shock genes in response to heat is accompanied by an equally rapid repression of the normal genes, that is, those active prior to the heat stress[226-230]. At the cytoplasmic level, heat shock mRNAs are preferentially translated while the normal mRNAs are stored in an inactive form. Superimposed on this transient activation of heat shock genes can be a developmental regulation: there are developmental stages or cell types in which a subset of heat shock genes is induced in normal physiological conditions and others in which heat shock genes are not inducible, even during heat shock. This heat shock gene can be regulated in a coordinate or noncoordinate way, they can be transiently or constitutively activated or they can be repressed. The heat shock genes are an attractive model system for studying the mechanism of transcriptional activation; how an individual gene can be regulated both transiently and during development, and how these two types of controls operate on the same gene are questions of great interest and discussion[231].

3 -INDUCTION OF THE HEAT SHOCK RESPONSE

The response of cells to heat shock is extremely rapid. Increases of the concentration of hsp mRNA from undetectable (less than one molecule per cell)[232] to a thousand molecules per cell can be observed within an hour of heat shock. Studies with inhibitors have shown that neither new protein nor new RNA synthesis are necessary for the transcriptional activation of heat shock genes[233]. Thus, the factor(s) involved must be preexisting.

Several heat shock genes have been cloned and sequenced. The induction mechanism of heat shock genes is enormously conserved; thus it was possible to introduce the Drosophila hsp 70 gene into mouse, monkey and frog cells and observe heat-shock induction of the foreign gene, concomitant with the endogenous heat shock response, despite the fact that the heat shock temperatures differ in these organisms[234-237]. Expression of the same gene in yeast was not properly regulated, suggesting that the yeast induction system has diverged from the one in higher eukaryotes[235]. Deletion analysis of the Drosophila hsp 70 gene established that gene sequences between -10 and -66 nucleotides upstream from the RNA start site are essential for heat shock induction. This region comprises the TATA box and a short conserved sequence element identified by Pelham[235], which can be found in a similar location in most Drosophila heat shock genes. It was shown that this element is a sufficient heat-shock regulatory element (HSE)[238]. Subsequent studies have identified a heat shock gene transcription factor or HSTF which specifically binds to HSE[239,240] and apparently correlates with transcription activity. A second protein was observed to be bound to the TATA box region and may generate DNase I- hypersensitive sites[239]. It is still not known whether HSTF undergoes a physical change during heat shock activation and how this change relates to its tight binding to the HSE. It is also not certain whether HSTF is loosely bound to proximal and distal HSEs before and to distal HSEs during heat shock.

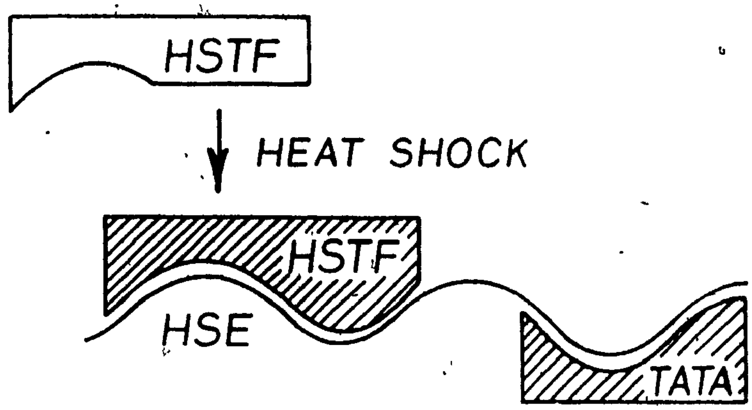
A working hypothesis for the transient activation of heat shock genes was described by Bienz[231] (**Figure 5**, System A). She proposed that the activation of heat shock genes depends on a common regulatory element, the HSE, in their promotors. The transcription factor, HSTF, is present in unshocked cells in an inactive state and may bind weakly to proximal and distal HSEs. Heat shock activates HSTF, and induces its tight binding to the proximal HSE and consequently transcription. The precise sequence of the HSE along with its location with respect to the TATA-box determine the efficiency of the induced transcription and the probability of a particular heat-shock gene being activated at very low active HSTF concentrations. Additional HSEs, located further away, can compensate for suboptimal promoter arrangements; they can increase transcription efficiency or ensure induction of a gene at limiting HSTF concentrations, possibly by weakly binding HSTF and increasing its local concentration. Although the HSE and HSTF are the key elements in this process, additional requirements such as the accessibility of the HSE in the chromatin and perhaps correct organization of the genes within chromosomal loops may be necessary for successful gene activation.

The developmental and non-coordinate regulation of heat-shock genes (**Figure 5**, System B) is caused by either differential promoter strength combined with suboptimal

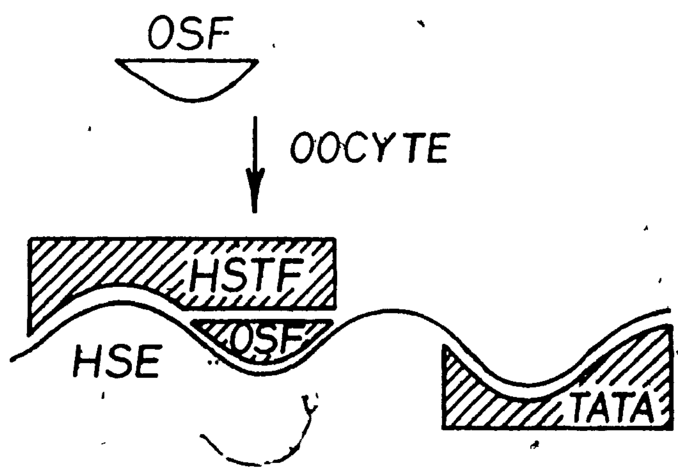
Figure 5

Models for transient and developmental activation of heat shock genes. System A shows the transient induction of heat shock genes which consists of a transcription factor (HSTF) that binds to a promotor element (HSE) when activated by heat shock. The developmental activation of heat shock genes, shown in System B, may operate in addition to or independent of System A. In this example the cell type-specific factor (OSF) modifies the activity of the transcription factor (HSTF) by allowing binding of the inactive transcription factor to the promoter. The TATA-box factor is required for both Systems A and B and binds to the TATA-box before the gene is activated possibly inducing accessibility of the adjacent promoter element. This figure was adapted from Bienz[231].

SYSTEM A



SYSTEM B



HSTF concentrations or the activity of additional cell type-specific factors. Such factors may be part of other activation systems and therefore be independent transcription factors (such as the ecdysone receptor). Alternatively, they may have an auxiliary function in modifying the activity of HSTF at HSE, for example, the putative Xenopus oocyte-specific factor, OSF. The sharing of elements for both the transient and developmental activation processes may reflect an economical strategy of evolution. Therefore, once the activation system A was set up, there was no need to evolve a complete second activation system B for a separate regulation of the same gene, but instead a single component B can overcome system A by modifying it[231].

4 - FUNCTION OF HSP'S

a -Structural

Within an organism, the hsp's can be subdivided into various classes differing in cellular localization and in their solubility. It is possible that different hsp's may have different cellular functions[227], a finding consistent with the reported differential induction of hsp's in various conditions[241,242]. For example, many hsp's are found associated with the nuclear preparations during heat stress. In Drosophila, these hsp's then return to the cytoplasm during recovery at normal temperatures[243]. Their distribution between cytoplasm and nucleus is dependent upon the temperature of the shock[244]. For both Drosophila[243,245], and Chironomus[241,246] it has been suggested that hsp's have a structural function either as constituents of the nucleoskeleton or matrix, or of the cytoskeleton. This is based on their presence in chromatin and on biochemical data. A structural function is compatible with the recent finding that the genes for the low molecular weight hsp's have considerable sequence homologies with crystallin genes[247]. Unfortunately the purity of the nuclear preparations from Drosophila cells is still questionable[227]. In addition, it has been suggested that in Tetrhymena, hsp's may be associated with the nuclear matrix[248]. In vertebrate cells hsp 68 can be observed in cytoskeletal elements[249,250]. Thus up to now, biochemical studies have not yielded unequivocal evidence concerning the function of hsp's[227].

b -<u>Thermotolerance</u>

The topic of the possible role for hsp's in thermotolerance in mammalian cells has recently been reviewed[251]. Studies on mammalian cells[252,253] and also in yeast[254], Drosophila[255], Calpodes[256], and Dictyostelium[257] have shown that cells exposed to sublethal temperatures develop a transient thermoresistance, which protects them from a second exposure at normally lethal temperatures. This phenomena, termed thermotolerance[253], has been divided into three processes[258]: an initial event (trigger); the expression of resistance (development); and the gradual disappearance of resistance (decay). While the molecular mechanisms underlying this transitory thermotolerance are unknown, various recent studies show a correlation between the development of thermotolerance and the synthesis of hsp's[259,260], while agents which induce hsp's similarly induce thermotolerance[261]. Finally, a strong relation between hsp induction and thermotolerance is suggested by findings in Dictyostelium where a mutant deficient in the expression of hsp is unable to develop thermotolerance[257]. Thus while no direct cause-effect relationship has yet been established between the synthesis of hsp and thermotolerance, there is a growing body of evidence suggesting the hsp's might take part in this process[227,262].

It is probable that this putative protective function of hsp's can be extended to other types of aggression different from heat. Thus, a mild heat pretreatment of Drosophila larvae protect them from phenocopy induction[255] and from the teratogenic effects of various drugs[263,264].

In studies with mouse lymphocytes it has been observed that the level of hyperthermia necessary to induce hsp synthesis *in vitro* and *in vivo* is of a magnitude (41°C[265]. This is the same temperature necessary to promote thermotolerance in mice[266], and also the temperature attained during febrile episodes in rabbits and humans[267]. From these findings it has been suggested that the *in vitro* and *in vivo* synthesis of hsp's by mouse lymphocytes represents a relevant physiological response which mammalian lymphocytes may normally undergo when submitted to periods of thermal stress[266].

It has been shown that when cultures of chicken embryo cells are heated briefly in the presence of either glycerol or D_2O, that the induction of hsp was almost completely blocked[268]. Possibly they protect the heat-sensitive proteins from irreversible denaturation by direct action or by generalized solvent effects and therefore no stress signals were generated and no hsp's synthesized[268]. Since D_2O and glycerol also have protective effects against thermal killing[269,270], possibly hsp's share similar stabilizing effects on stress-sensitive cellular components[271].

5 -HEAT SHOCK GENES AND DEVELOPMENT

The observation that in many organisms heat shock related genes are expressed only during certain stages of cell development suggests that hsp's may be involved in differentiation.

For example, specific heat shock-like proteins have been observed only during the late erythroblast stage of human erythroid maturation[272]. During embryogenesis in mice two proteins identical to hsp 68 and hsp 70 are present at the two-cell embryo stage but not at the one-cell-stage[273]. In many organisms, proteins which might correspond to hsp's appear during early embryogenesis concomitant with the onset of transcription of the embryonic genome. For example, in Drosophila this has been described at the blastoderm stage[274]. It might be necessary for the embryo to acquire rapidly a high level of hsp 70 for the onset of the embryonic genome expression. Perhaps this could explain why conditions such as hyperthermia, or exposure to ethanol, which stimulate hsp synthesis, promote the parthenogenetic activation of the mouse oocytes[273].

A heat shock response was observed in parasitic protozoa (Trypanosoma brucei, Leishmania major) when shifting from their poikilothermic insect adapted vector (promastigotes) to the homeothermic mammalian host adapted vector (amastigotes)[275]. This observation, along with the demonstration that a shift in temperature *in vitro* induced the differentiation of Leishmania major from promastigotes to amastigotes provides additional evidence that heat shock genes are involved in differentiation[275]. A possible role for hsp synthesis in the control of the eukaryotic cell cycle has been suggested based on the synthesis of two hsp's (73,56) in the G_O phase[276]. The induction of hsp's in G_O cells is distinct from that in heat stress cells in two respects: it is more transient; and G_O cells synthesize only high molecular weight hsp's[277]. In addition, a heat shock resistant mutant of *saccharoyces cerevisiae* in which the hsp genes are expected to be constitutively expressed, was observed to have altered growth with an elongated G_1 period[276].

6 - HEAT SHOCK AND HYPERTENSION

Heat stress has been observed to affect blood pressure in a number of studies. In control Wistar rats, along with rats receiving ethanol in their drinking water, long term heat stress significantly elevated mean systolic blood pressure[278]. In experiments with SHR it has been shown that they are more sensitive to noxious stimuli (heat stress, ether vapors) than Sprague Dawley, as observed in altered patterns of change in growth hormone and prolactin pituitary content, and adrenal ascorbate and cholesterol[279]. In addition, SHR are not able to thermoregulate as well as WKY during warming procedures[280] as shown by higher SHR basal colonic temperatures, and an increased incidence of death. Other investigators have observed similar basal colonic temperature for WKY and SHR however, SHR temperatures increased significantly more than WKY throughout the two hour heat stress period[281]. An increased incidence of death has also been observed for SHR on clonidine therapy and heat stress[282].

Lethal heat sensitivity is also observed in another genetically hypertensive model, the spontaneously hypertensive mouse[283]. In our laboratory, hypertensive and normotensive mice of the Schlager strain were heated in a box for 5 min, five times a week, at 40°C, over a period of

30 days, while control hypertensive and normotensive animals were handled identically but without being heated. At the end of the experiment the control hypertensive mice remained hypertensive however, a significant decrease in blood pressure was observed for the heated hypertensive mice from Day 1 to Day 30 (from 127 to 90 mmHg)[26].

It has also been shown that the genetic hyperthermia-stress syndrome is preceded and accompanied by an intense peripheral vasoconstriction and by hypertension[284], and that heat stress-susceptible pigs are hypertensive[285] Thus it is possible that in at least three genetically based hypertensive models, heat sensitivity and the effect of altered thermotolerance on blood pressure may be related to hypertension.

III - METHODS AND MATERIALS

A - ANIMALS

The Wistar Kyoto and Spontaneously Hypertensive Rats (250-300g, 15 weeks old) used for breeding throughout these studies were obtained from Charles River Canada. Rats were housed on a 12-hour light and dark cycle with food and water available ad libitum. Rats were numbered by ear punching and body weight, heart rate and systolic blood pressure, routinely recorded.

1 - BLOOD PRESSURE DETERMINATIONS

Systolic blood pressure (mmHg) and heart rate (beats/min) were determined for WKY and SHR rats by the tail cuff method (programmed electro-sphygmomanometer and pneumatic pulse transducer Pe-300, Narco Bio-Systems Inc.). Although this is an indirect method of determination, it has been shown to have an excellent correlation with direct systolic pressure measurements in the carotid artery[286]. The procedural guidelines for the determination of blood pressure by the indirect tail cuff method[287] were followed. Four readings were recorded for each animal, and mean values determined. Once rats had become familiarized with this procedure, blood pressure and heart rate were determined three times during two weeks prior to breeding.

2 - BREEDING

The WKY and SHR breeding schedules were identical. Breeding stocks for WKY and SHR rats consisted of 4 males and 12 females each. The WKY and SHR female stocks were divided into 3 groups (4 rats each), and each group bred within 1-2 weeks of each other to provide a fairly continual supply of newborns. Female rats were rebred no sooner than two weeks after parturition when pups were sacrificed.

B - IN VIVO ^{3}H-TDR INCORPORATION

1 - LABELLING WITH ^{3}H-TDR

Newborn rats (less than 24 hours old) were weighed and injected with methyl-^{3}H-thymidine (0.66 µCi/g, i.p.; specific activity 20 Ci/mM; New England Nuclear). Animals were maintained at 30°C for a period of 6 hours after the injection, then decapitated; the hearts, kidneys, liver, adrenal glands, and aortas were removed. Right and left kidneys were combined, as were adrenal glands, and results were expressed as a single value per animal. The tissues were immediately frozen in liquid nitrogen, weighed, and stored at -70°C until assay.

2 - TISSUE CONTENT DETERMINATIONS

The tissues were homogenized in 1 ml of distilled water with a Vari-Mix II-M (Caulk Company) three times for 10 sec, at high speed. Aliquots of homogenate were taken for estimations of protein by the method of Lowry[288] and DNA by the method of Burton[289]. A 100 µl aliquot of homogenate was placed in a scintillation vial containing 10 ml of scintillation fluid for determination of tritium content (Beckman LS-230 liquid scintillation counter). In addition, a 100 µl aliquot of DNA extract, prepared from homogenate by acid precipitation with 0.5 N perchloric acid and hydrolysis by heating for 20 min at 100°C, was counted for ^{3}H-TDR incorporation into DNA. For determinations with aorta and adrenals it was necessary to pool 3-4 organs per assay. All data presented for these organs have been adjusted to express values per organ. From values of total organ tritium activity (^{3}H-total) and incorporation of ^{3}H-TDR into DNA (^{3}H-DNA) several parameters were calculated for all WKY and SHR organs. The equations used for these calculations are listed below.

1) Accumulation = ^{3}H-total/organ weight (CPM/mg)
2) Incorporation into DNA = (^{3}H-DNA/^{3}H-total) x 100 (%)
3) Specific Activity of DNA = ^{3}H-DNA/total organ DNA (CPM/µg)

Data are presented as means with standard errors. The number of newborns studied was 40 for WKY and 34 for SHR. Statistical comparisons were made by unpaired student's t-test. Statistical significance in all studies was considered present at p values of less than 0.05%.

C - In Vitro Cardiocyte Studies

1 - MATERIALS

The materials used for cell culture are listed by manufacturer. All chemicals were of Analytical Reagent Grade. The water used for preparing solutions was glass bidistilled.

Aldrich Chemical Company: HEPES sodium salt, D(+) glucose. Amersham: phase combining system scintilation fluid. J.T. Baker Chemical Company: (ethylenedinitrilo) tetraacetic acid. BDH Chemicals: glacial acetic acid, sodium chloride, perchloric acid 70%. Becton Dickonson Labware: 24-well multiwell culture dishes. Corning Glass Works: 25 cm^2 tissue culture flasks. Difco Laboratories: gelatin. Fisher Scientific: ethanol. GIBCO: trypsin (1:250), fetal calf serum, calf serum, Dulbecco's modified Eagle medium 340-1600, L-glutamine, Ham F12 nutrient mixture 430-1700. Millipore Corporation: nitrocellulose filters (0.22μm,0.45μm pore size). New England Nuclear: methyl-^3H-thymidine (specific activity 20 Ci/mM) Sigma: penicillin-G, streptomycin sulfate.

2 - PREPARATION OF PRIMARY CARDIOCYTE CULTURES

All cell culture procedures were performed under sterile conditions. Methods routinely used in our laboratory to maintain sterilization and aseptic conditions are well documented[290,291].

The WKY and SHR cardiocyte cultures were prepared simultaneously. The starting material for each culture consisted of pooled ventricles from 8-15 pups collected from 1-4 litters. The parents' number, blood pressure, pulse rate, along with the number of pups taken for culture, their age, and litter size were recorded. The technique used for preparing primary cardiocyte culture was described by Cantin et al[292].

a -~~Dissection~~

Newborn rats were decapitated and immediately placed in a beaker of 70% ethanol. Ventricles were removed and placed in a small petri dish containing 5 ml of "washing medium" consisting of Hanks balanced salt solution (BSS)[293] without calcium and magnesium, with 2% penicillin-streptomycin solution[294] and buffered with 10 mM HEPES to pH 7.4[295]. Ventricles were minced and washed two more times.

b -Cell Dissociation

The minced tissue was transferred to a 100 ml bottle containing 10 ml of 0.1% trypsin[296] in BSS without calcium and magnesium, buffered with 10 mM HEPES to pH 7.4. The bottle was then placed into a 37°C agitating water bath for a dissociation process of seven steps; the first two steps each lasting 10 min, the third and forth 15 min, and the fifth to seventh 20 min. After each step, the minced ventricles were aspirated 20 times in a 10 ml pipette to disperse the cells, placed in a 15 ml centrifuge tube, and mixed with 3 ml of fetal calf serum (FCS) to neutralize enzyme activity[291]. For each step 10 ml of fresh trypsin solution was added to the bottle. The suspensions of cells were centrifuged at 400 x G for 5 min and each pellet resuspended with 1 ml of complete medium consisting of Dulbecco's modified Eagle's medium[295], buffered with 10 mM

HEPES to pH 7.4 and supplemented with 10% FCS[297], 1% 200mM L-glutamine[295] and 1% penicillin-streptomycin[294]. All serum used for cell culture had been heat inactivated at 57°C for 30 min. The cells from dissociation steps two through six were pooled and cells counted.

c -Counting

Two methods for estimating cell suspension concentration were used in our laboratory. The most convenient and practical method was with a hemacytometer (AO Brightline, Scientific Instruments[291]). Cell counts were also often determined with a Coulter electronic cell counter (Coulter Electronics[291]). Four counts were recorded for each sample and total cell number per suspension determined.

d -Plating

Cells were plated at an initial density of 2×10^6 to 3×10^6 cells on 25 cm^2 gelatinized culture flasks in 5 ml complete medium. A gelatinized substrate has been found to be beneficial for the culture of muscle[298]. To prepare flasks, 4 ml of gelatin (5 mg/10 ml sterile water) was added to each flask for three hours. The excess gelatin was removed and flasks remained in the laminar flow hood overnight to dry.

Once cells were plated the complete medium was renewed after 24 hours and then routinely twice a week. Cultures were incubated at 37°C with 95% O_2, 5% CO_2. Cell attachment and morphology was routinely verified by phase contrast microscopy (Nikon).

e -Selective Plating

To obtain cultures enriched in myocardiocytes, a modification of Kasten's original technique of selective plating[296], described by Cantin et al[292] was used. This procedure is based on Kasten's observation that in a suspension of cardiocytes, the non muscle cells (endothelial cells, fibroblasts) attach to a substrate surface within several minutes. In contrast, myocardiocytes remain rounded for approximately 15 hours before they exhibit membrane extension and subsequent attachment to the surface. The final suspension of cells from the dissociation steps two through six were plated on 25 cm^2 gelatinized culture flasks at 37°C for 30 min. This was repeated three times to allow as many nonmyogenic cells as possible to attach to the gelatinized surface. The final myocardiocyte-enriched supernatent was centrifuged at 400 x G for 5 min and then plated.

f -Washing and passaging cells

Cells were washed with Dulbecco's phosphate buffered saline (PBS)[299] without calcium and magnesium, with 0.2% glucose. When colonies of myocardiocytes became confluent, the cells were detached by incubation in PBS without calcium and magnesium containing 0.02% EDTA and 0.05% trypsin, at 37°C for 4 min. Cells were aspirated with a pasteur pipette off the flask surface and collected in a centrifuge tube.

Cells were centrifuged 5 min 400 x G, counted and replated at the desired plating density.

3 - Cell Number

a - Growth rate in F12 5% PDS-CM and complete medium

Four day-old WKY and SHR cultures were trypsinized and replated on large cluster wells each at an initial plating density of 8 x 10^4 cells. Cells were maintained in 2 ml of F12, 5% PDS-CM medium (medium used to prepare quiescent cultures) or complete medium, which were replenished after 24 hours of initial plating, and then every third day. On days 3, 6, 9, 12, and 14 in culture, cells were trypsinized from three wells and a 1 ml aliquot taken from each well for cell count determinations with the Coulter counter. For cells maintained in complete medium the remaining 1 ml of cell suspension was centrifuged for 5 min at 400 x G and resuspended in 0.5 ml of distilled water, three 150 µl aliquots were taken for creatine phosphokinase (CPK) estimations (P-L Biochemicals Colorimetric Calibration Set).

b - Plating Efficiency

To compare plating efficiency for WKY and SHR cultures, cardiocytes were plated at an initial plating density of 2 x 10^4 cells on petri dishes and maintained in complete medium for 24 hours. Cells were then trypsinized and cell counts determined with the Coulter Counter.

4 - DETERMINATION OF DNA SYNTHESIS WITH ^{3}H-TDR INCORPORATION

a - Establishing Quiescence

To render WKY and SHR cultured cardiocytes quiescent prior to determination of DNA synthesis and cell cycle length, confluent cells were trypsinized and plated at 4 x 10^4 cells/ml in 24-well cluster dishes in complete medium. After 24 hours, when cell attachment was complete, the medium was changed to Ham's F12 (F12)[295] supplemented with 5% plasma (PDS-CM). The PDS-CM was prepared from normal human plasma and then passed through a CM Sephadex C-50 column to remove residual platelet derived growth factor activity[300]. The cells were incubated in this medium for 48 hours to synchronize the cells into G_0 or G_1 phase of the cell cycle.

b -The 36 hour time course

Cell cycle length was determined by 36 hour time courses, this procedure, the scheme shown in **Figure 6**, was originally established in our laboratory for vascular smooth muscle cells[301] and adapted for cardiocytes. Fresh F12 and 5% PDS-CM containing 5% FCS was added to synchronized WKY and SHR cardiocytes to stimulate cells for 8, 12, 16, 20, 24, 28, and 36 hours. Basal unstimulated control cells were maintained in F12 and 5% PDS-CM. At the end of each time period, the medium was changed to DME that contained 0.5 µCi/ml ^{3}H-TDR for a pulse label of two hours. Each well was then washed with 1 ml isotonic saline to remove excess ^{3}H-

Figure 6

The scheme for 36 hour time course with cardiocytes from newborn rats.

Trypsinized cells

↓

Cluster wells (4×10^4 cells/well)

↓

Hours	24	48	8	12	16	20	24	28	36
Solutions	Complete medium with 5 FCS	Ham's F12 with 5 PDS-CM				Ham's F12 with 5% PDS-CM and 5% FCS			

↓

2 hour time pulse DME and ^{3}H-TDR

↓

DNA extraction

TDR and the cells were fixed with 1 ml ethanol:acetic acid (3:1) for 10 min. The fixative was removed and the cells were washed with 1 ml water. Acid-insoluble material was precipitated by incubation with 1 ml cold (4°C) 0.5M perchloric acid (PCA) for 15 min. After further washing with 1.0 ml cold 0.5M perchloric acid to remove all nonincorporated ^{3}H-TDR, DNA was extracted into 1.5 ml PCA by heating to 90°C for 20 min. The PCA, containing solubilized DNA, was transferred to scintillation vials containing 10 ml PCS; the radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer (LKB Wallac). The rate of DNA synthesis was expressed as counts per minute (cpm) ^{3}H-TDR incorporated into DNA per well, and all determinations were in quadruplicate. Time courses for WKY and SHR were repeated with three different cell preparations. Data are presented as means with standard errors. Comparisons were made by unpaired student's t-test. Time courses were also completed for two additional normotensive strains Wistar (WAR), and Sprague Dawley (SDR). Results were also calculated as fractions of the highest peak of DNA synthesis (cpm of highest peak = 1), and compared for all rat strains.

5 - AUTORADIOGRAPHY

Autoradiography was completed for samples of asynchronous and synchronized primary cultures of WKY and SHR cardiocytes, along with samples of synchronized cultures stimulated to grow with 10% FCS for 16 and 28 hours. Cells were grown on coverslips which were placed on the bottom of small petri dishes. Four coverslips were used for each test time. All samples received a 1 hour pulse label with ^{3}H-TDR (0.5 μCi/ml). Cultures were then washed three times with isotonic saline, three times with F12, and again three times with saline to remove nonincorporated ^{3}H-TDR. Cells were fixed with 95% ethanol and formaldehyde (9:1) for 30 min, washed with water, and dried at room temperature overnight. Coverslips were mounted on glass slides and dipped in K5 emulsion (Ilford Nuclear Research, Ciba-Geigy) diluted with water 1:1. The coated slides were dried in the dark room overnight and then transferred to light tight black boxes containing dessicants. The cultures were exposed to the emulsion for two weeks at 4°C[302]. Slides were developed with D-19 for 4 min (Kodak), fixed with 20% sodium thiosulfate for 3 min, rinsed with water, and stained with toluidine blue for 1 hour or with periodic acid Schiff technique[302]. Slides were then rinsed with water and air dried and covered with an additional cover slip. Slides were observed with a phase-contrast microscope and photographed. The labelling index was derived from the ratio of labelled cardiocytes to total cardiocytes. A minimum of 100 cardiocytes per region was counted, 10 regions were counted for each test time. Only darkly labelled nuclei were counted. Means with standard errors were determined and comparisons were made by the unpaired student's t test.

6 - FLOW CYTOMETRY

a - Flow cytometer

Flow cytometric analysis of WKY and SHR cardiocytes were performed using an Epic V Flow cytometer (Coulter Electronics). Flow cytometry allows for determination of cell size by light scatter properties and DNA content per cell by fluorescence of fluorochrome bound DNA[303-305]. Cells in suspension are placed into a stream by a hydrodynamic focusing effect. The cells then pass through a pinpoint laser beam which provides a powerful beam of coherent, monochromatic light which can be tuned to several different wavelengths. Two major events occur when the cell is exposed to the laser beam. First the light is scattered by the cell in 360°, however the light scattered in the forward direction along the axis of the laser beam is approximately proportional to cell size. The magnitude of this parameter, known as forward angle light scatter, is directly proportional to the size of the particle provided the particle is a homogeneous sphere. Fluorescence, the second event, occurs when a fluorochrome containing cell absorbs the laser light at the incident wavelength and then reemits the light at a longer wavelength. The phenomena occurs rapidly (within 10^{-8} seconds); the emitted light is at a lower energy and, thus, a different color. The fluorescent light is collected through an optical detector system located orthogonally to the laser beam. The greater the amount of light emitted from the cell, the larger the electrical impulse. This signal is converted to a digital signal and can be recorded as a data point in a frequency distribution histogram.

b -Cell preparation

Samples of freshly dissociated cardiocytes (cells which were not placed in culture), synchronized samples (cultured cells synchronized for 48 hours in F12, 5% PDS-CM), and samples of stimulated cardiocytes (synchronized cells stimulated to grow with F12, 5% PDS-CM and 5% FCS for 0, 12, 14, 16, 20, 24 and 28 hrs) were suspended in isotonic saline with cell numbers adjusted to similar cell densities for WKY and SHR samples. Cells were then fixed; a proper fixation procedure had not been previously established for cardiocytes and therefore several fixatives (4% paraformaldehyde, 70% ethanol and 1% glutaraldehyde) were compared. Cells were centrifuged 400 x G for 10 min to remove fixative, resuspended in isotonic saline, and recentrifuged. The saline was replaced with 0.5% pepsin in saline at pH 1.5 and samples incubated for 30 min at 37°C. The reaction was stopped with a buffer containing 100 mM TRIS, 100 mM NaCl, and 8.4% HCl at pH 7.4 in distilled water. The cells were incubated with RNAse (200 units/ml saline) for 30 min at 37°C, since the DNA fluorochrome used binds indistinguishably to RNA as well as DNA, RNAse was required in the procedure to obtain specific DNA staining. Cells were then washed with saline and centrifuged at 400 x G for 10 min. Propidium iodide 50 µg/ml saline was added for at least 20 min and cells were measured with the flow cytometer at an excitation wavelength of 488 nm, and emmission wavelength greater than 570 nm.

To determine if tissue-related differences in DNA distributions existed, freshly dissociated cells derived from newborn WKY heart, spleen, blood, and lung were prepared under identical conditions and compared with flow cytometric analysis.

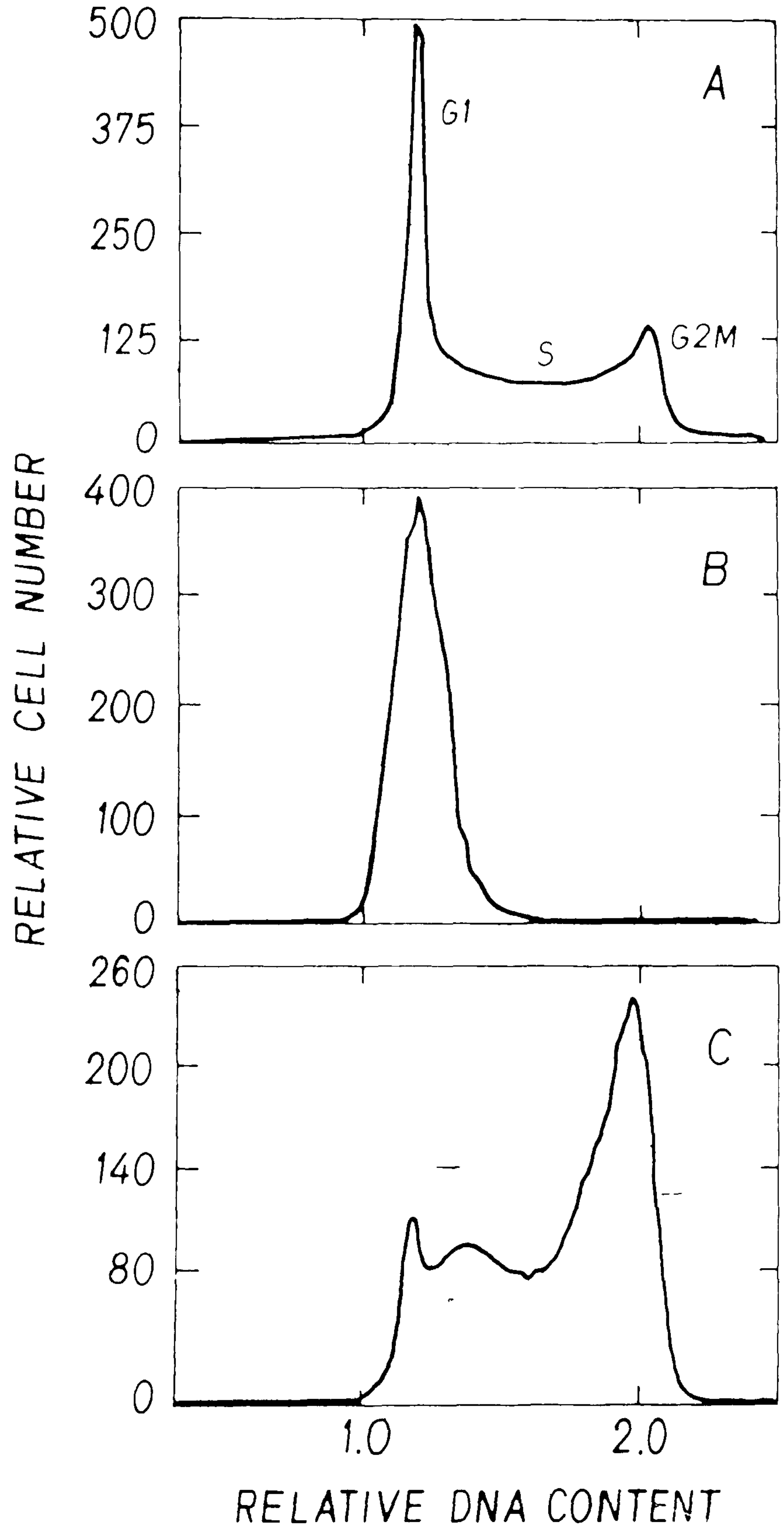
c -DNA distribution interpretation

Flow cytometric DNA analysis is based on the following principles: (a) cells in different stages of the cell cycle have different but predictable amounts of DNA, (b) flurochromes are available that stoichiometrically bind to DNA, and (c) normal cells are euploid and have a known amount of DNA[303].

A DNA distribution typical of an asynchronous, homogenous cell population is illustrated in **Figure 7A** and is characterized by several distinctive "landmarks"[305]. The large peak at unit relative DNA content is due to G_1 phase cells, the smaller peak at twice this DNA content is due to G_2-M phase cells, (no distinction can be made because cells in both G_2 and M phases have the same DNA content), and the continuum between is due to cells at intermediate points in

Figure 7

Interpreting DNA distribution histograms. **Panel A** - the DNA distribution for an asynchronous exponentially growing cell population. This distribution is characterized by the "landmark" G_1 and G_2-M phase peaks and the intermediate S phase region. **Panel B** - the DNA distribution for a cell population synchronized in late G_1 and early S phase, note that the landmark features mentioned in panel A are missing. **Panel C** - the DNA distribution for the cell population of panel B taken at a later time. This population has desynchronized sufficiently so the G_1 and G_2-M landmarks are visable but not enough so that the population has become as asynchrounous as panel A. This figure was adapted from Gray et al[305]



500
375
250
125
0
A
G1
S
G2M
400
300
200
100
0
B
260
200
140
80
0
C
RELATIVE CELL NUMBER
1.0
2.0
RELATIVE DNA CONTENT

S phase producing increasing amounts of DNA as they transit this phase. In addition, DNA distribution sequences are sensitive indicators of synchrony in cycling cells, changing in response to changes in the distribution of cells around the cycle as shown in **Figures 7B and C.** To estimate the fraction of cells in the G_1, S, and G_2-M regions, the spectrum is divided into three regions corresponding to the three phases. The fraction of cells in each phase is then estimated by planimetric measurement of the area of each region. The percentages of cells in each cell cycle phase were recorded as means with standard errors and each reading was taken with an aliquot of 1×10^4 cells.

7 - DOSE RESPONSE OF SERUM

The rate of DNA synthesis was determined for WKY and SHR cardiocytes following a 24 hour exposure to 0, 1, 5, 10, 15 and 20% fetal calf and calf serum by ^{3}H-TDR incorporation into DNA as previously described. Dose responses were completed for three different cell preparations. Each dose was tested in quadruplicate. Data are presented as means with standard errors. Comparisons were made by unpaired student's t test.

8 - PREPARATION OF BLOOD COMPONENTS

The scheme for preparation of plasma derived serum (PDS), whole blood serum (WBS), and platelet extract (PE) described by Ross et al[300] is shown in **Figure 8.** Age and sex matched WKY and SHR adult rats were anesthesized with

Figure 8

Preparation of plasma derived serum (PDS), whole blood serum (WBS) and platelet extract (PE) from adult WKY and SHR.

CITRATED WHOLE BLOOD

5 ML		3 ML
CENTRIFUGATION AT 500 X G, 5 MIN TO REMOVE RED BLOOD CELLS		ALLOW TO CLOT WITH CA^{2+} AT $37^{\circ}C$ FOR 30 MIN
CENTRIFUGATION AT 1800 X G TO SEPARATE PLATELETS		CENTRIFUGATION AT 1800 X G
ALLOW TO CLOT WITH CA^{2+} AT $37^{\circ}C$ FOR 2 HRS	RESUSPEND PLATELETS IN TRIS	HEAT AT $56^{\circ}C$ FOR 30 MIN
CENTRIFUGE AT 1800 X G	FREEZE AND THAW	CENTRIFUGATION AT 1800 X G
HEAT AT $56^{\circ}C$ FOR 30 MIN		
CENTRIFUGATION AT 1800 X G	CENTRIFUGE [illegible]0,000 X G	
PDS	PL	WBS

0.1ml/100g body weight Somnotal i.p. and exsanguinated via carotid artery catheterization. Blood was drawn into syringes containing 1:10 volumes of 60 mM EDTA in isotonic saline to drawn blood. Whole blood from each rat was divided into two aliquots: 4 ml into plastic centrifuge tubes for preparation of PDS and PE; and 3 ml in 15 ml glass corex tubes for WBS.

To prepare WBS, whole blood was recalcified with 20 mM $CaCl_2$ and allowed to clot at 37°C for 2 hours. The clotted blood was then centrifuged at 1800 x G for 20 min at 25°C, the serum was decanted from the clot and collected in glass centrifuge tubes.

To prepare PDS, the aliquot of blood was centrifuged at 500 x G for 5 min at 4°C to remove red blood cells, and again at 1800 x G for 20 min to sediment platelets. The plasma was then allowed to clot with 20 mM $CaCl_2$ at 37°C for 2 hours and centrifuged at 1800 x G for 20 min at 25°C to remove the fibrin clot. The PDS was collected in a glass centrifuge tube.

To prepare PE, platelets were dispersed in 1 ml of 10 mM TRIS in a plastic centrifuge tube, and homogenized by quickly freezing and thawing three times by placing the tube in 95% ethanol and dry ice. The resulting platelet lysate was centrifuged for 10 min at 30,000 x G at 4°C, and collected in a glass centrifuge tube.

The PDS, WBS and PE were heat inactivated at 56°C for 30 min and centrifuged at 1800 x G for 10 min at 25°C.

Blood components were stored at -70°C until assay and protein content was determined with the method of Lowry[288]. Growth stimulating potential of blood components was determined by rate of DNA synthesis with ^{3}H-TDR incorporation as previously described, once synchronized, cardiocytes were exposed for 24 hours to 50-100 µl samples of PE, PDS, or WBS. Blood components were also tested on a more homogenous population of cultured cells, vascular smooth muscle cells derived from WKY aorta.

9 - BEATING RATE OF MYOCARDIOCYTES

a - Culture Flask Perfusion Chamber

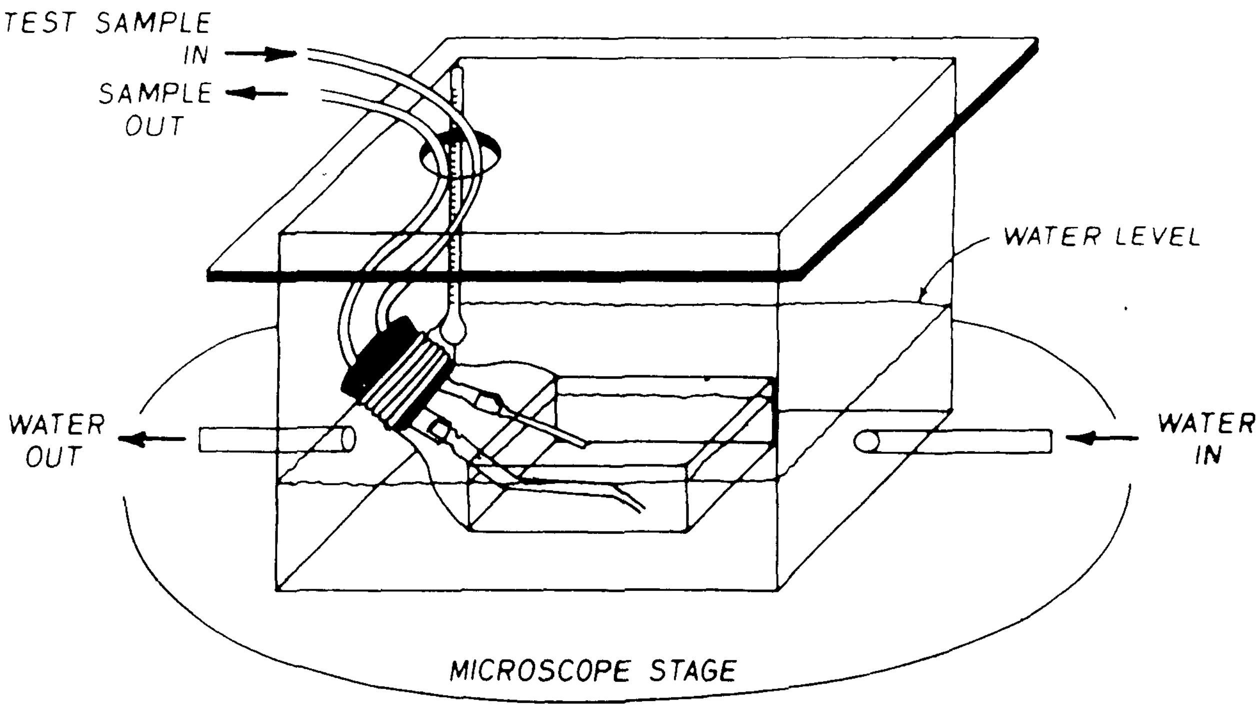
To study the spontaneous beating rate of myocardiocytes we designed a culture flask perfusion chamber which is illustrated in **Figure 9**. A culture flask containing a monolayer of cardiocytes fits into the base of the perfusion chamber. With the use of a peristaltic pump (Buchler Instruments) water warmed to the desired temperature was pumped into the chamber surrounding the culture flask. Test samples could also be pumped into and out of the culture flask. The chamber was placed on the stage of a phase-contrast microscope (Nikon) where beating rates (beats/15 sec) of cell clusters were counted visually, and beats/min calculated.

b - Chronotropic Effect of Temperature

Beating rate was estimated for 5 to 6 day old WKY and SHR myocardiocyte-enriched cultures at 25, 27, 30, 31, 34,

Figure 9

Culture flask perfusion chamber used to observe beating rates of myocardiocyte clusters.



CULTURE FLASK PERFUSION CHAMBER

and 37°C. Flasks were fitted into the perfusion chamber once the appropriate temperature was obtained, and equilibrated for 15 min before beating rates were counted. Flasks were removed from the chamber after each test and maintained at 37°C until the next test temperature was obtained in the perfusion chamber. At least 10 myocardiocyte clusters/flask were counted. A total of 20 flasks derived from two preparations of WKY and SHR myocardiocytes were tested and data presented as means ± SE, and compared with student's t-test.

10 - THERMOLABILITY OF CARDIOCYTES

Thermolability was determined for WKY and SHR cardiocytes in suspension and cultured on flasks. Cell viability was determined by a dye exclusion test with trypan blue[293].

a -Cardiocytes in Suspension

Cardiocytes in suspension were prepared by trypsinizing confluent cultures and resuspending cells in complete medium (1×10^6 cells/ml). The stock suspensions were kept in the 37°C incubator and 0.5 ml aliquots transferred to test tubes for heat exposure. Test tubes were placed in a water bath at the desired temperature and duration, then removed from the bath and 0.5 ml of trypan blue solution (0.4% trypan blue in PBS with 0.2% glucose) was added to each sample for 2 min at room temperature. A visual count of the number of cells with blue stained nuclei (nonviable cells), along with the total number of cells was estimated with the hemacytometer. Four counts were recorded for each sample and percent mortality determined.

$$\% \text{ Mortality} = \frac{\text{Number of cells stained blue}}{\text{Total number of cells}} \times 100$$

b -<u>Cardiocytes in Culture</u>

Flasks of cultured cells in 5 ml of complete medium were placed in a water bath for the determined heat exposure. Flasks were removed from the bath and the complete medium was replaced with 4 ml of trypan blue solution for 2 min at 37°C. The dye was then removed and the cells washed two times with 5 ml PBS with 0.2% glucose solution, and 5 ml of complete medium was added to the flask for 5 min at 37°C. The flask was placed under the phase-contrast microscope with the eyepiece reticle in place and percent mortality determined for 5 different fields of the flask. If an additional heat exposure was required the flask was placed in the incubator for 5 min prior to testing.

c - <u>Thermotolerance</u>

To determine if heat tolerance could be induced, 24 and 48 hr cultures were divided into two groups: control and test flasks. Test flasks received an initial heat exposure at 48°C for 10 or 15 min, test and control flasks were then maintained at 37°C for 6 hrs, and then heated at 55°C for 20 min and percent mortalities determined.

11 - PREPARATION OF Ca^{2}-TOLERANT CELLS

Two additional cell dissociation procedures were attempted for preparation of Ca^{2+}-tolerant cells. Cardiocytes were prepared by the following modifications of the procedures described by (a) Haworth et al[306], and (b) Altschuld et al[307].

a) Excised newborn hearts were placed in a petri dish containing 5 ml of Krebs Henseleit bicarbonate medium (Buffer A) containing: 118 mM NaCl, 4.8 mM KCl, 25 mM Na_2HCO_3, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 11 mM glucose at 37°C for 10 min, hearts were washed three times. Buffer A was then replaced with 5 ml of Buffer B (Buffer A without $CaCl_2$, with 0.002% phenyl red) for 5 min, hearts were washed two times. The hearts were then transferred to a 100 ml bottle containing 10 ml Buffer B with 20 mg collagenase. The bottle was placed in an agitating 37°C water bath for 20 min. The softened hearts were removed from the bottle, minced with scissors in a small petri dish, and returned to the bottle now containing 10 ml of Buffer B and collagenase with 1 mM $CaCl_2$ and 0.1 mg trypsin. The bottle was placed in the agitating water bath for four 20 minute dissociation steps. The cells collected after each dissociation step were filtered through a 300µm nylon mesh in centrifuge tubes. The mesh was rinsed with 5 ml Buffer C (Buffer A without $CaCl_2$, and bicarbonate replaced with 25 mM HEPES, pH 7.4) at room temperature. Cells were centrifuged for 5 min at 400 x G, and pellet washed twice by resuspension in 5 ml Buffer C and centrifugation. Cells were plated in complete medium as previously described.

b) Excised newborn hearts were placed in a petri dish containing 5 ml Krebs-Ringer bicarbonate medium (Buffer A) containing 120 mM NaCl, 5 mM KCL, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 10 mM glucose containing 1mg/ml BSA, and 25 µm $CaCl_2$ and washed three times at 30°C for 5 min. Hearts were placed in a 100 ml bottle containing 10 ml Buffer A and 10 mg collagenase. The bottle was placed in an agitating 30°C water bath for 20 min. The softened hearts were removed from the bottle, minced with scissors in a small petri dish, and returned to the bottle for four 20 min dissociation steps. The cells collected after each dissociation step were transferred to plastic centrifuge tubes on ice and centrifuged at 400 x G for 5 min. Cells were resuspended and washed two times with Krebs-Henseleit medium identical to Buffer A except 16mM Na_2HPO_4 and 11 mM glucose were added and calcium and BSA omitted (Buffer B). Cells were layered over the same buffer to which 4% BSA was added in plastic centrifuge tubes, and sedimented by centrifugation for one minute at 100 x G. Cells were resuspended in Buffer B and preincubated for 10 min at 30°C prior to culture in complete medium. Beating rates (beats/min) and percentage of beating clusters/flask (number of beating clusters/total number of clusters per flask) x 100 were determined for five flasks each of WKY and SHR cardiocytes from both dissociation procedures. Growth rates were also compared by cell number counts on days 3, 6, 9 and 12 in culture.

IV RESULTS

A - INCORPORATION OF METHYL-^{3}H-THYMIDINE INTO DNA OF WKY AND SHR ORGANS IN VIVO

1 - Basic Data for Parents

Table 1 shows mean systolic blood pressure, pulse rate and body weight for WKY and SHR parent rats. Systolic blood pressure was significantly higher, and pulse rate slightly faster, for the SHR mothers and fathers than in WKY.

2 - Newborns

a - Body and organ weight

At birth, SHR were heavier than WKY rats ($p<0.001$), (5.2 ± 0.4 g, n=34 vs 4.2 ± 0.4g, n=40). The observed higher SHR body weight was not associated with any significant differences in litter size (9.3 ± 0.6 vs 8.0 ± 0.9 pups/litter). Total organ weights for all SHR organs studied were significantly greater than in WKY (**Table 2**). Relative organ weight (mg/g body weight) however, was significantly higher ($p<0.001$) only for SHR hearts and kidneys (17% and 24% higher respectively) (**Figure 10**). In contrast, relative liver weight for SHR was significantly lower than WKY (-8%). Relative weights of WKY and SHR adrenal glands were similar, and although relative aorta weights were higher for SHR, differences did not reach statistical significance.

T A B L E 1

Mean systolic blood pressures, pulse rates, and body weights for WKY and SHR rats used for breeding

PARENTS	WKY	SHR
Number of Mothers	[illegible]	[illegible]
Blood pressure (mmHg)	112 ± 2	1[illegible]8 ± 4***
Pulse rate (beats/min)	430 ± 10	[illegible] ± 1[illegible]
Body weight (g)	227 ± 9	21[illegible] ± 3
Number of Fathers	1	4
Blood pressure (mmHg)	122 ± 1	200 ± 1***
Pulse rate (beats/min)	430 ± 10	480 ± 10
Body weight (g)	317 ± 5	377 ± 2***

***$p < 0.001$

TABLE 2

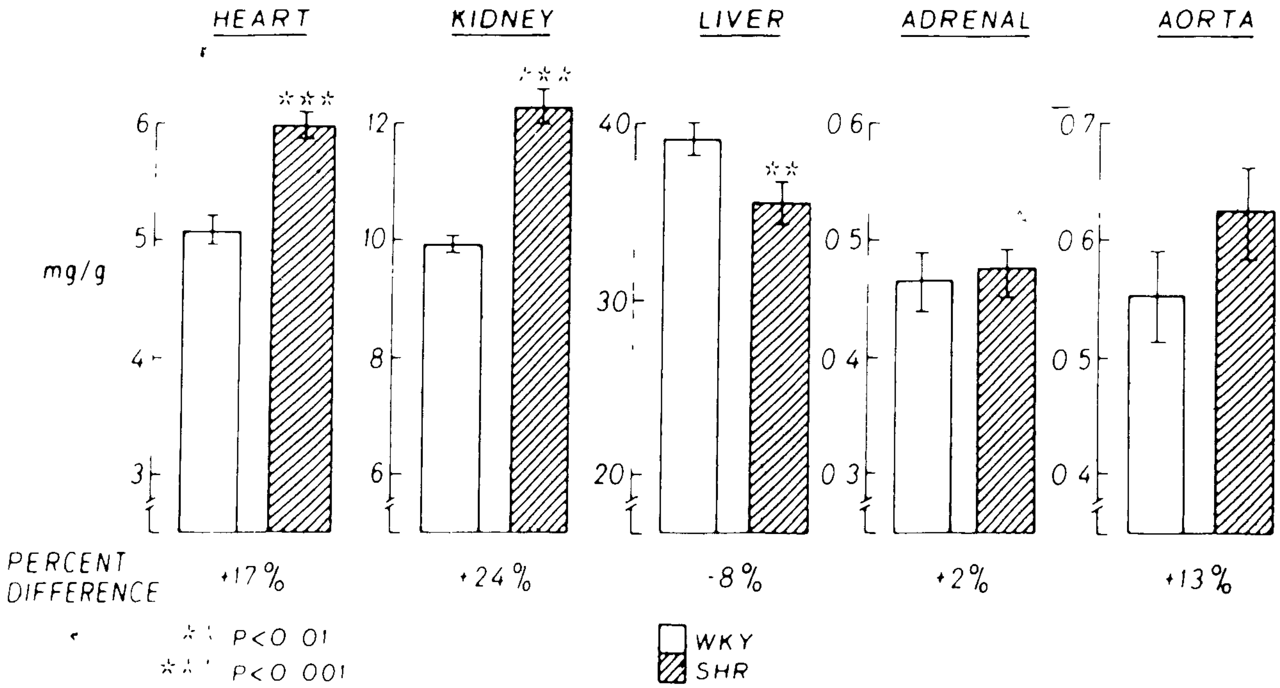
Total organ weights for newborn WKY and SHR

Organs	Organ weight (mg)	
	WKY	SHR
Heart	21.5 ± 0.6	30.9 ± 0.6***
Kidney	42.2 ± 1.1	63.5 ± 1.4***
Liver	167.0 ± 5.2	187.0 ± 4.7**
Adrenal	1.95 ± 0.10	2.48 ± 0.10***
Aorta	2.31 ± 0.15	3.22 ± 0.19***

** $p<0.01$
*** $p<0.001$

Figure 10

Relative organ weights (organ to body weight ratio) for newborn WKY and SHR organs. Data are means ± SE, WKY n=34, SHR n=40


HEART
KIDNEY
LIVER
ADRENAL
AORTA
mg/g
PERCENT DIFFERENCE
+17%
+24%
-8%
+2%
+13%
** P<0 01
*** P<0 001
WKY
SHR

b -Protein and DNA content

The higher total weight observed for all SHR organs was also reflected by significantly higher total organ protein, with the exception of adrenals (**Table 3**). Relative protein content, however, was significantly higher only in SHR heart (+44%) and in kidney (+22%). This finding is consistent with the significantly higher relative organ weight, again observed only for heart and kidney. Protein content per mg organ weight was significantly higher for SHR heart, but was not significantly different for other organs.

Higher total organ DNA was observed for SHR heart, kidney, liver, and perhaps aorta (**Table 4**). Significantly higher relative DNA content (+37%) was observed for SHR kidney ($p<0.001$) and heart (+17%). Relative DNA content for SHR adrenal was significantly lower than WKY but not significantly different for WKY and SHR aortas. The DNA content per mg organ weight was significantly higher for SHR liver, but was not significantly different for other organs.

c -Total organ tritium activity and ^{3}H-TDR incorporation

Total organ tritium content and incorporation of ^{3}H-TDR into DNA reflected organ size, and for WKY and SHR was highest in liver, then kidney, heart, and adrenals and aortas (**Table 5**). The percentage of the injected tritium label present in organ homogenate and in DNA extract was higher for all SHR organs, except liver. Total organ tritium accumulation, incorporation into DNA, and specific

TABLE 3

Protein contents for newborn WKY and SHR organs

	Total organ protein (mg)		Protein/body weight (mg/g)		Protein/organ weight (mg/mg)	
	WKY	SHR	WKY	SHR	WKY	SHR
Heart	1.5 ± 0.1	2.6 ± 0.1***	0.36 ± 0.03	0.52 ± 0.02***	0.07 ± 0.01	0.08 ± 0.01**
Kidney	3.3 ± 0.2	4.9 ± 0.2***	0.78 ± 0.04	0.95 ± 0.03***	0.08 ± 0.01	0.08 ± 0.01
Liver	21.4 ± 0.7	25.0 ± 0.7***	5.03 ± 0.14	4.78 ± 0.15	0.13 ± 0.01	0.08 ± 0.01
Adrenal	0.20 ± 0.03	0.19 ± 0.02	0.046 ± 0.008	0.036 ± 0.004	0.07 ± 0.01	0.08 ± 0.01
Aorta	0.12 ± 0.02	0.23 ± 0.04*	0.029 ± 0.004	0.044 ± 0.008	0.06 ± 0.01	0.08 ± 0.01

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

DNA content for newborn WKY and SHR organs

	Total organ DNA (μg)		DNA/body weight (μg/g)		DNA/organ weight (μg/mg)	
	WKY	SHR	WKY	SHR	WKY	SHR
Heart	44.1 ± 2.9	66.5 ± [illegible].2***	[illegible]0.30 ± 0.65	12.06 ± 0.77	1.97 ± 0.14	2.12 ± 0.14
Kidney	138.8 ± 7.3	224.0 ± 9.[illegible]***	32.11 ± 1.57	43.87 ± 2.26***	3.31 ± 0.16	3.58 ± 0.17
Liver	471.5 ± 18.8	597.8 ± 17.8***	111.32 ± 4.33	112.21 ± 4.50	2.86 ± 0.10	3.22 ± 0.09*
Adrenal	5.92 ± 0.86	4.82 ± 0.66	[illegible].63 ± 0.26	0.93 ± 0.13*	3.11 ± 0.40	2.12 ± 0.41
Aorta	4.77 ± 0.53	5.7[illegible] ± [illegible]	[illegible].12 ± 0.12	1.10 ± 0.21	2.32 ± 0.41	2.11 ± 0.50

* p <0.05, *** p <0.001

TABLE 5

TOTAL ORGAN TRITIUM CONTENT AND INCORPORATION OF
(METHYL-^{3}H) THYMIDINE INTO DNA OF WKY AND SHR ORGANS

	^{3}H-total (cpm/organ)x10^{3}		^{3}H-DNA (cpm/organ)x10^{3}	
ORGANS	WKY	SHR	WKY	SHR
Heart	4 94±0 40 (1%)	9 52±0 60 (2%)***	1 64±0 15 (0 33%)	3 29±0 24 (0 66)***
Kidney	23 54±1 83 (5%)	41 00±1 96 (8%)***	9 72±0 80 (2%	20 30±1 11 (4%)***
Liver	70 76±4 09 (14%)	70 40±4 48 (14%)	53 4±3 54 (11%)	78 90±4 07 (16%)***
Adrenal	0 70±0 10 (0 14%)	1 24±0 18 (0 25%)*	0 30±0 05 (0 06%)	0 55±0 10 (0 11%)*
Aorta	0 57±0 05 (0 11%)	1 25±0 21 (0 25%)**	0 20±0 04 (0 04%)	0 53±0 10 (0 11%)**

*p<0 05
**p<0 01
***p<0 001

Tritium content and (% of injected radioactivity) for newborn WKY and SHR organs
Left panel shows total organ tritium content and right panel shows (methyl-^{3}H) thymidine incorporated into DNA

activity of DNA for all organs are shown in **Table 6.** Significantly higher organ accumulation was observed only for the heart (34% over WKY). Incorporation into DNA was significantly higher for SHR kidney (+24%) and liver (+55%). Specific activity of DNA however, was significantly elevated only in SHR heart (+33%) and kidney (+39%) (**Figure 11**).

d -Discussion

Cardiomegaly is present whether the newborn SHR body weight is similar to[108,109] lower than[1,110] or higher than (present study) that of WKY controls. The finding of similar litter size for WKY and SHR eliminates the possibility that SHR organs are larger merely because of smaller litters. This is important to note in view of studies which show that neonatal rats reared in groups of progressively fewer pups show a higher rate of division of ventricular muscle cells[53,54]. Our data also include values from newborns taken for assay before suckling thus, eliminating any effect of quantity of milk intake on growth rate.

The higher total weights observed for all SHR organs studied reflects in part the higher body weight of the newborns in the present study. It is the determination of organ to body weight ratio, however, which clearly shows the excessive relative growth only in SHR heart and kidney. The significantly higher relative protein content, again observed only for SHR heart and kidney, also reflects organ

TABLE 6

TOTAL ORGAN ACCUMULATION INCORPORATION AND SPECIFIC ACTIVITY OF (METHYL-^{3}H) THYMIDINE FOR NEWBORN WKY AND SHR ORGANS

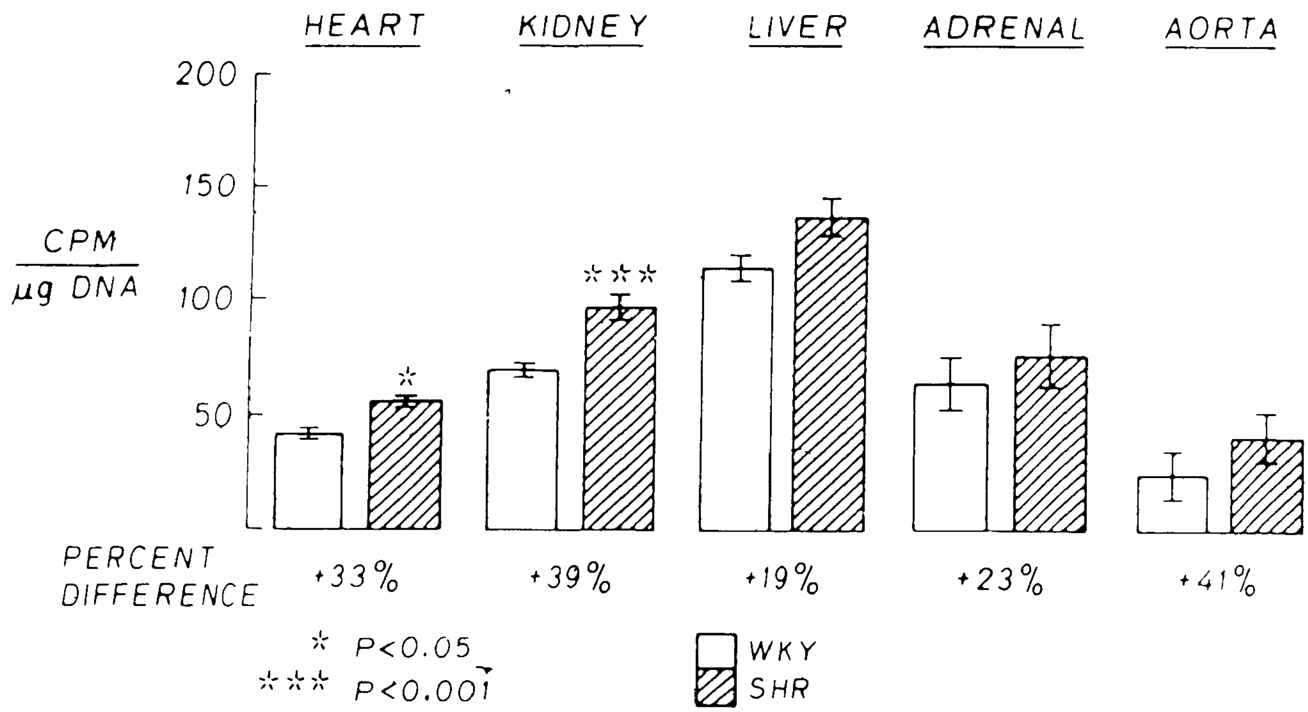
		HEART	KIDNEY	LIVER	ADRENAL	AORTA
ACCUMULATION (cpm/mg)	WKY	230±18	566±44	458±35	326±55	299±30
	SHR	308±18**	625±37	387±28	458±59	344±55
INCORPORATION (%) INTO DNA	WKY	34±2	41±1	76±4	44±3	33±3
	SHR	35±2	51±2***	118±5***	44±3	42±3
SPECIFIC ACTIVITY OF DNA (cpm/μg)	WKY	42±4	69±4	114±7	61±12	29±4
	SHR	56±4*	96±6***	136±10	75±14	41±5

**$p<0.01$

***$p<0.001$

Figure 11

The specific activity of DNA derived from ^{3}H-TDR and percent difference between WKY and SHR newborn organs. Data are means + SE, WKY n=34, SHR n=40.


HEART
KIDNEY
LIVER
ADRENAL
AORTA
200
150
100
50
CPM / μg DNA
PERCENT DIFFERENCE
+33%
+39%
+19%
+23%
+41%
* P<0.05
*** P<0.001
WKY
SHR

enlargement. This abnormality is not a result of higher tissue water content since protein per mg organ weight for SHR heart and kidney was higher or similar to WKY. This finding, in addition to the elevated relative DNA contents observed for SHR heart and kidney, is consistent with previous studies and confirms the presence of hyperplasia in newborn SHR heart and kidney. An increase in DNA per cell was not indicated since DNA per mg organ weight was similar for WKY and SHR heart and kidney.

Total organ tritium accumulation, incorporation into DNA, and specific activity of DNA provided evidence of several differences in SHR organs. In an in vivo study of this nature it is important to acknowledge that many factors other than rate of DNA synthesis will influence these parameters. Such factors include the availability of labelled thymidine for DNA synthesis, which will depend on distribution of blood vessels, rate of blood flow, and permeability of cell membranes. In addition, the incorporation of thymidine into DNA proceeds by a sequence of phosphorylation steps followed by its assembly along with other nucleoside triphosphates into DNA. Therefore, the activity of the enzymes involved in the biosynthesis of DNA (e.g., thymidine kinase, thymidylate kinases, DNA polymerases) and the presence of factors required for enzyme activity (ATP, Mg^{+2}) and for thymidine degradation (thymidine phosphorylase) will greatly affect incorporation. Many cellular defects in SHR have already been described, such as cell membrane permeability to monovalent ions[308], Ca^{+2} uptake and binding and Ca^{+2}-ATPase activity in SHR monocytes[309,310], cyclic nucleotide and adenylate cyclase systems[311,312] and calmodulin levels[313].

The demonstration of significantly higher specific activity of DNA derived from (methyl-^{3}H) thymidine for SHR heart and kidney, in the present study, is consistent with, but does not prove, the hypothesis of enhanced cellular proliferation in these organs. This is also evident from significantly higher values of incorporation into DNA in SHR kidney. The inability to detect a higher incorporation in SHR heart may be due to the altered total tritium accumulation for this organ. The higher total organ tritium activity in SHR heart may be masking the increase in incorporation.

Assays for aortas and adrenals were difficult because of the organ size, but some differences for SHR were observed. Protein and DNA contents for SHR adrenals were lower than WKY despite similar relative organ weights. The possibility of increased fat in SHR adrenals may be responsible for this discrepancy. The finding of similar thymidine specific activities for WKY and SHR adrenals is consistent with results from histoautoradiographic studies showing similar labelling indexes for adrenal medullas of 20-day-old SHR and WKY fetuses[314]. For SHR aorta, higher relative weights and protein contents may reflect a slight hypertrophy for this organ. Specific activity of DNA was higher for SHR aorta, although cellular hyperplasia was not apparent from DNA content values. A growth abnormality in SHR aorta has already been suggested based on observations of higher ornithine decarboxylase activity in cultured aortic smooth muscle cells from SHR when compared to WKY[115].

The pattern of abnormalities of organ size, protein, and DNA contents, particularly for heart and kidney, is similar for newborn SHR and other models of essential hypertension[1] (**Figures 1 and 2**). Different abnormalities were observed for renal and experimental models of hypertension. These findings suggest that a developmental defect may occur in essential hypertension during the fetal period of organogenesis resulting in an altered growth of cardiovascular organs which may in turn be related to the expression of the hypertensive phenotype.

The increase in DNA content and specific activity of DNA *in vivo* are strong indicators that an enhancement in DNA synthesis is present in newborn SHR heart. It is unclear whether this is evidence that at birth cardiocytes are growing faster in SHR, hence, a shorter cell cycle length than WKY; or that an exaggerated cellular proliferation occurred in utero but had already normalized at birth, the higher specific activity of DNA is therefore a reflection of a larger number of cells synthesizing DNA at any given point. These questions are addressed with quantitative cell cycle analysis of WKY and SHR cardiocytes *in vitro* described in the next chapter. Results of this study have recently been published[112].

B - In Vitro Cardiocyte Studies

1 -Cell Culture

The cell yields for dissociation procedures were 3 x 10^6 - 4 x 10^6 cells per heart. The freshly dissociated heart cells were spherical in shape. Cell cultures were heterogenously comprised of myocardiocytes, fibroblasts and endothelial cells. Once plated, fibroblasts began attachment to the substrate within 15-30 min, myocardiocytes remained rounded for many hours before exhibiting signs of membrane extension and subsequent attachment to the surface. Within 24 hours most cells had attached, were flattened and irregular in shape. The myocardiocytes were thicker than the non-muscle cells and most were beating spontaneously within 24 hours in culture. Cultures became confluent within 4 to 6 days. Once a confluent monolayer was reached cardiocytes continued to grow forming a thick sheet of cells; if not passaged, cells eventually lifted from the flask surface forming large spherical aggregates of cells.

2 - Cell Number

The WKY and SHR cardiocytes could be maintained in F12 medium with 5% PDS-CM for up to 12 days in culture (**Table** 7). In this medium the cell population remained almost unchanged. No significant differences were observed for WKY and SHR on each day. Cardiocytes maintained in complete medium (**Table 8**) increased in cell number up until 12 days in culture; on Day 14 cultures were confluent and cells

TABLE 7

CELL NUMBER OF WKY AND SHR CARDIOCYTES

— CULTURED IN HAM'S F12 AND 5% PDS-CM

days in culture	WKY	SHR
0	31 7 ± 0 8	31 7 ± [illegible]
3	34 1 ± 1 2	33 9 ± [illegible]
6	36 8 ± 1 0	37 0 ± 1 [illegible]
9	35 2 ± 0 8	35 4 ± 0 [illegible]
12	32 5 ± 1 0	32 [illegible]

TABLE 8

CELL NUMBER OF WKY AND SHR CARDIOCYTES

CULTURED IN COMPLETE MEDIUM

days in culture	WKY	(CPK I/1)	SHR	(CPK I/1)
0	82 2 ± 8 0	(422 ± 3 5)	82 2 ± 8.1	(408 ± 3 7)
3	233 4 ± 11 0	(259 ± 2 8)	230 9 ± 11 2	(260 ± 2 6)
6	427 2 ± 11 5	(349 ± 2 8)	467 9 ± 12 0	(336 ± 2 8)
9	757 6 ± 10 7	(278 ± 3.0)	748.0 ± 10 5	(280 ± 3 2)
12	892 8 ± 10.0	(250 ± 3.0)	890 4 ± 11 0	(262 ± 3 0)
14	672.2 ± 10 6	(217 ± 2.8)	669.1 ± 10 0	(210 ± 3 0)

Cell number ± SE x 10^3, WKY and SHR n=3 began to detach. Again no significant differences were observed for WKY and SHR. Creatine phosphokinase (CPK) levels for WKY and SHR cardiocytes were similar on each day and decreased with time in culture.

3 - Discussion

The F12, PDS-CM medium was used routinely in our laboratory to synchronize cells in the G_1-G_0 phase of the cell cycle prior to DNA synthesis determination. As shown by the consistently low cell numbers, PDS-CM contains only competence factors and therefore maintained cell viability but was unable to stimulate cells to initiate DNA synthesis. In contrast, complete medium containing fetal calf serum provides cells with the necessary progressive factors to stimulate cells to enter S phase. Determinations of CPK, an enzyme specific to muscle cells, was used as an indication of muscle cell concentration for cardiocytes. The similar CPK values observed for WKY and SHR cultures may be a reflection of similar contents of myocardiocytes within the heterogenous cell population. From this study the use of PDS-CM medium for synchronizing cardiocytes and fetal calf serum for stimulating DNA synthesis was substantiated. The cell numbers for WKY and SHR cultured with FCS reflected similar growth rates for WKY and SHR cardiocytes.

4 - The 36 hour time course

Table 9 shows the basic data for the parent rats used in this study. The five day-old cultures obtained from WKY

TABLE 9

BASIC DATA FOR NORMOTENSIVE AND HYPERTENSIVE RATS

	Strain			
	WKY	WAR	SDR	SHR
MOTHER				
Blood Pressure (mmHg)	115 ± 1	121 ± 2	122 ± 2	172 ± 2
Pulse (beats min)	420 ± 10	380 ± 20	360 ± 10	420 ± 10
Weight (g)	229 ± 14	237 ± 6	254 ± 14	208 ± 13
FATHER				
Blood Pressure (mmHg)	121 ± 2	130 ± 3	121 ± 1	200 ± 1
Pulse (beats min)	400 ± 20	360 ± 10	380 ± 20	420 ± 30
Weight (g)	307 ± 17	303 ± 8	431 ± 6	387 ± 5

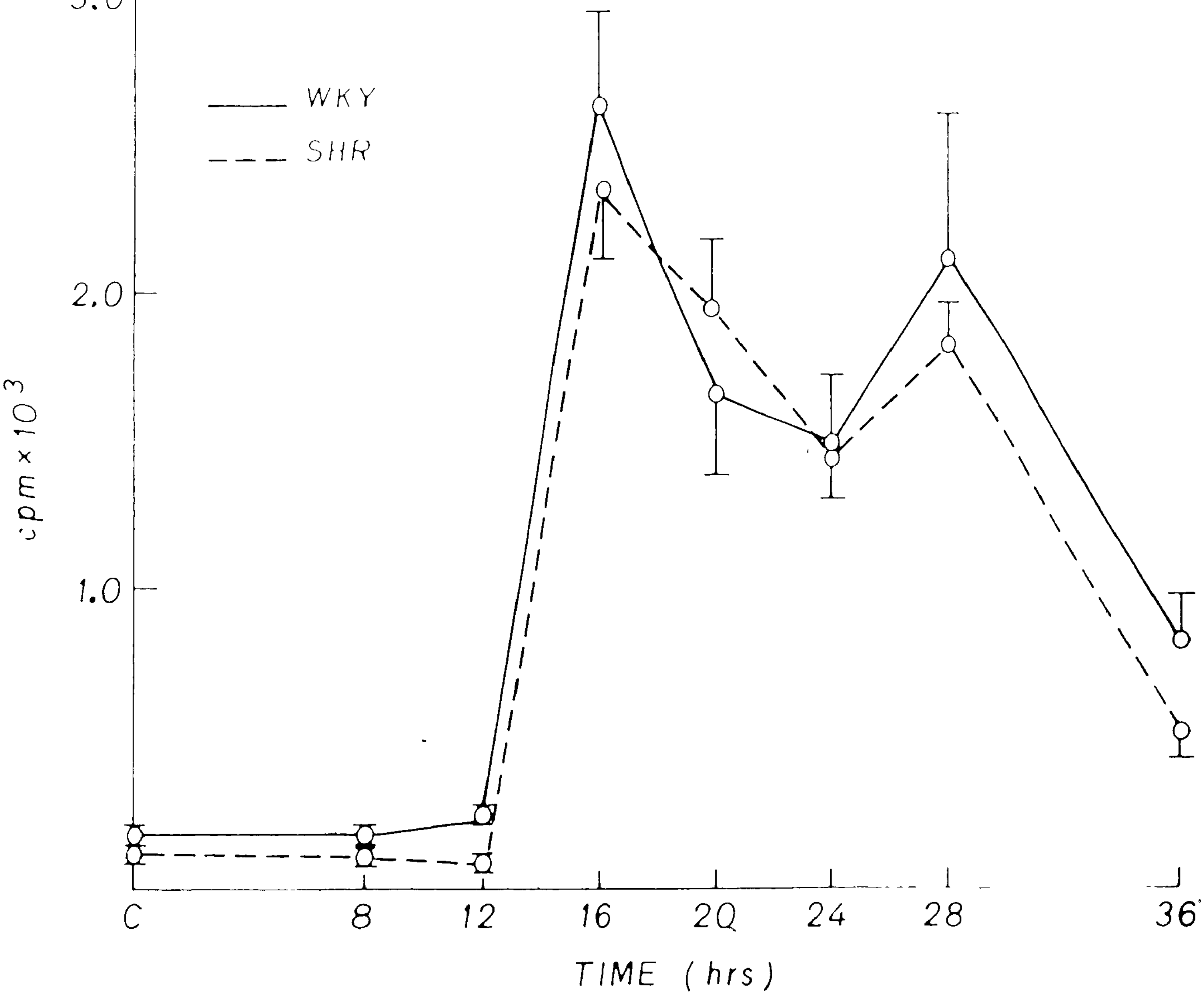
Data are means ± SE, WKY n=3, SHR n=3, WAR n=2, SDR n=2 and SHR newborns had similar patterns of DNA synthesis (**Figure 12**). The control, basal unstimulated levels of DNA synthesis assayed in serum-free medium were very low confirming that cells were stationary. A lag time of 12 hours was necessary before stimulated cells initiated DNA synthesis. Two peaks of DNA synthesis were observed, the largest peak at 16 hours and a smaller peak at 28 hours. By 36 hours the level of DNA synthesis had decreased toward basal levels. Significantly lower cpm/cluster well were observed for SHR at 8 and 12 hour time points. Relative DNA synthesis patterns showed that SDR had similar patterns to WKY and SHR (**Figure 13, Table 10**). The WAR pattern of DNA synthesis differed slightly from the other strains, there was a gradual increase in ^{3}H-TDR incorporation into DNA after 16 hrs, and the highest peak was observed at 28 hrs.

5 - Discussion

Patterns of DNA synthesis for cardiocytes using the time course procedure described in this study have not been previously reported. Growth rates of cardiocytes from WKY and SHR have not been compared before. However, a study with 6 day-old cardiocyte cultures from 3-4 day-old Wistar rats using a similar procedure was completed by Frelin et al[316]. In their study, cells were synchronized for 48 hours in serum-free medium and then shifted to medium with 20% serum, a continuous rather than pulse label of ^{3}H-TDR was used for DNA synthesis determinations. They observed a lag

Figure 12

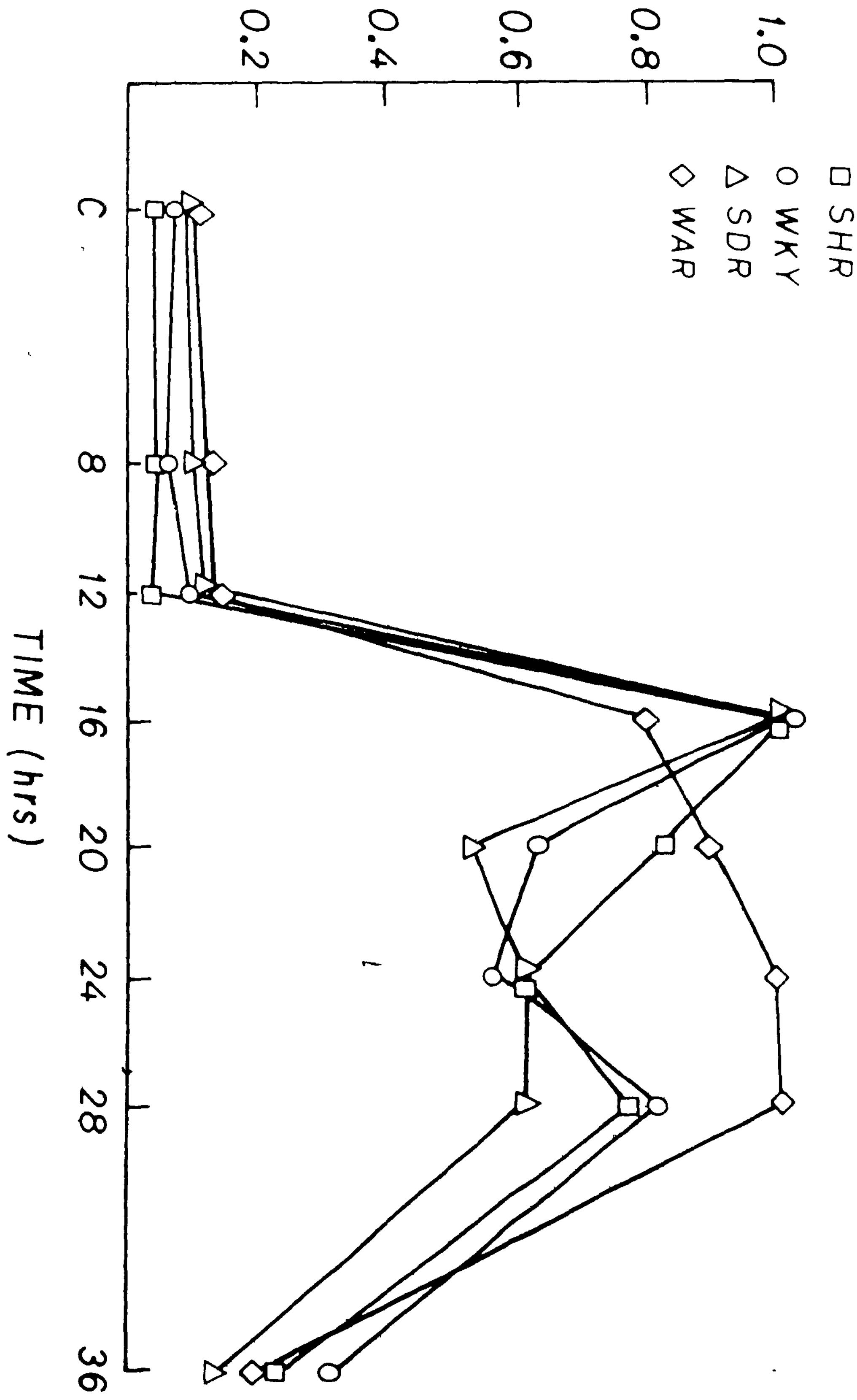
The 36 hour time course for WKY and SHR cardiocytes. Data are mean cpm per cluster well ± SD from three different preparations each; n = 16 for WKY and SHR.



3.0
2.0
1.0
cpm × 10³
WKY
SHR
C
8
12
16
20
24
28
36
TIME (hrs)

Figure 13

Relative DNA synthesis for 36 hour time courses for newborn cardiocytes from normotensive (WKY, WAR, SDR) and hypertensive rats (SHR).



□ SHR
○ WKY
△ SDR
◇ WAR
1.0
0.8
0.6
0.4
0.2
C
8
12
16
20
24
28
36
TIME (hrs)

TABLE 10

36 HOUR TIME COURSES FOR CARDIOCYTES

STRAIN	HOURS 0	8	12	16	20	24	28	36
WKY	18[illegible]±21	166±19	228±26	2612±331	1645±191	1467±228	2109±488	801±171
SHR	101±23	100±12	83±12	2334±227	1923±257	1413±116	1809±149	501±94
WAR	104±[illegible]	128±6	127±8	822±52	923±53	1032±86	1040±36	194±12
SDR	129±8	142±16	181±15	1370±113	733±33	823±55	831±54	174±10

Data are mean cpm per cluster well ± SE

WKY n=16 SHR n=16 WAR n=12 SDR n=8

period of 12 hours and then a steady rise in incorporated radioactivity up to 15 hours, which then increased more rapidly and leveled off after 24 hours of incubation. Mitotic figures were observed after the burst of DNA synthesis, and they suggested that the cells were blocked in early G_1 phase at the time of shifting to 20% serum. They estimated the time required for cells to complete the cell cycle to be about 30 hours. The time course of ^{3}H-TDR incorporation into DNA of cardiocytes obtained from newborn WAR observed in our study is compatible with their findings.

The explanation for the presence of two peaks of DNA synthesis at 16 and 28 hours for the WKY, SHR, and SDR cardiocytes is unclear. Several possibilities may be responsible for this observation. For example, cultures of cardiocytes are heterogenous, the two peaks of DNA synthesis may therefore reflect two cell types with different cell cycle lengths. The mammalian heart is composed of muscle (myocardiocytes) and nonmuscle (fibroblasts, endothelial cells) cells. The relative population of these cell types varies with age[317], but myocardiocytes in the neonatal rat heart are approximately 60% of the cells. Proteolytic enzyme digestion of neonatal rat heart yields a cell suspension that contains a slightly greater number of myocardiocytes (70-80%), than that observed *in vivo*[318,319]. The proliferative activity of myocardiocytes in culture had been overlooked for many years primarily because of their low mitotic rate. However *in vivo* studies of ^{3}H-TDR incorporation into DNA of rat myocardial cells have shown that a sustained hyperplastic growth phase exists for these cells prior to 4 days of age[33-35]. *In vitro* studies have shown that muscle and nonmuscle cardiac cells have different growth potentials, the length of mitosis for nonmuscle cells has been estimated to be 2.5 hours, and 6 hours for muscle cells. The highly proliferative potential of nonmuscle cells is further demonstrated by their ability to overtake cultures[99,319,321]. Kasten[320] has shown that primary cultures of cardiocytes with an initial contamination of 5% fibroblasts will change in the ratio of myocardiocytes to fibroblasts from 9.5:1 to 1:1 after only 4 days in culture. This overgrowth may also be reflected by our observation of lower CPK contents obtained from older cultures of WKY and SHR cardiocytes (**Table 8**). Myocardiocytes and fibroblasts appear to coexist in culture in a metabolically functional synergism as shown by Schroedel et al[321] who compared the metabolism of mixed heart cell cultures with enriched cultures of myocardiocytes and fibroblasts. They observed that mixed cultures had increased rates of glucose utilization, cellular protein, along with greater resistance to stress of serum and insulin deprivation. Frelin[322] has also shown that growth rates were much faster for mixed cardiocyte cultures than enriched muscle or nonmuscle cells.

Another possible explanation for the two peaks of DNA synthesis in the 36 hour time course is that myocardiocytes which are beating in culture may have an altered growth rate. Kasten[120] has shown with cinematography, that contracting myocardiocytes do undergo mitosis. The contracting cells were observed to maintain their beating pattern at prophase, contractions became weaker at metaphase and ceased at anaphase. It was not until several hours later when the daughter nuclei had fully reformed that contractions resumed. This mitosis was not always followed by cytokinesis which resulted in binucleated cells. Disorganization of myofibrils along with competition for energy stores were suggested as possible factors for the change in beating rate during myocardiocyte mitosis. Further discussion of myocardiocyte beating rates along with comparisons of beating rates for WKY and SHR cultures are discussed in section E. The second peak of DNA synthesis observed from the 36 hour time course may simply be a result of cells entering a second cell cycle starting again at the 24 hr time point, or cells may have already become desynchronized.

The time course of ^{3}H-TDR incorporation only measures cells which are actively synthesizing DNA in S phase, it gives no information on the cell types involved, the labelling index (proportion of cells synthesizing DNA to total cell population), or the proportions of cells in G_1, M, or G_2 phases of the cell cycle. In the following section additional methods of autoradiography and flow cytometry used to further investigate the growth rates of WKY and SHR cardioctyes are discussed. Time points 16 and 28 of the cell cycle were compared with autoradiography to determine which cell types were in S phase. A potential difference in plating efficiency was also investigated for these cells since lower cpm/cluster well was observed for SHR at time points 8 and 12, a possible indication of lower plating efficiency for SHR cells.

Despite the limitations of the ^{3}H-TDR incorporation procedure, the results provided evidence that under these culture conditions cardiac cells derived from SHR had identical patterns of DNA synthesis with two normotensive rat strains, WKY and SDR. These results were consistent with the similar growth rates for WKY and SHR cardiocytes cultured in complete medium determined by cell number.

6 - Plating Efficiency

Cell number was lower for SHR cardiocytes than WKY within 24 hours of initial plating ($p<0.001$), ($3.02 \times 10^4 \pm 0.37$, $n=60$ vs $3.71 \times 10^4 \pm 0.34$, $n=66$). The increase in cardiocyte cell number over initial plating density (2.14×10^4) after 24 hours in culture was +73% for WKY and +41% for SHR.

7 - Autoradiography

As a result of the formation of large, high cell density, beating colonies of myocardiocytes in culture, it was difficult to count myocardiocytes and determine labelling indices. With our procedure the labelling indices could only be determined for cardiocytes that were more evenly dispersed and separate from the large clusters of cells.

Asynchronous cultures of WKY and SHR cardiocytes (**Figures 14 and 15**) had similar labelling indices (24.0 ± 2.8% and 23.7 ± 2.5% respectively). A 48 hour exposure to serum-free medium was successful in synchronizing cells, (**Figures 16 and 17**) with labelling indices of only 1.4 ± 0.3% for WKY, and 1.3 ± 0.3% for SHR. No significant differences in labelling indices were observed between WKY and SHR following serum stimulation, after 16 hours (**Figures 18 and 19**) 28.5 ± 2.5% of WKY cells and 29.4 ± 2.3% of SHR cells were labelled; after 28 hours 21.0 ± 2.6% and 23.6 ± 2.4% of WKY and SHR cardiocytes were labelled

8 - Discussion

The labelling indices estimated for the asynchronous WKY and SHR cultures were similar to findings by Hollenberg et al[54] from autoradiographs of newborn SDR rats. In their study the newborn rats received an intraperitoneal injection of ^{3}H-TDR and after 1 hour 18.8 ± 1.1% of cardiocytes had picked up significant amounts of label. The majority of labelled cells observed by Hollenberg and in the present study were non muscle cells. This is also consistent with autoradiographic analysis of two day-old cardiocyte cultures from 2-3 day-old WAR following a 48 hour pulse of ^{3}H-TDR completed by Kasten[296]. Kasten observed a labelling index of 65% for non-muscle cells and 2% for myocardiocytes in

Figure 14

Autoradiograph of asynchronous primary WKY cardiocytes cultured for 28 hours in complete medium followed by a 1 hour pulse label of ^{3}H-TDR. The ^{3}H-TDR is incorporated into newly synthesized DNA and therefore darkly stained nuclei are only found in cells actively synthesizing DNA in S phase. Magnification 2,400 x

Figure 15

Autoradiograph of asynchronous primary SHR cardiocytes cultured for 28 hours in complete medium followed by a [illegible] hour pulse label of ^{3}H-TDR. Magnification 1,200 x

Figure 16

Autoradiograph of primary cultures of WKY cardiocytes synchronized for 48 hours in Ham's F12 and 5% PDS-CM followed by a 1 hour pulse label of ^{3}H-TDR. Magnification 1,200 x

Figure 17

Autoradiograph of primary cultures of SHR cardiocytes synchronized for 48 hours in Ham's F12 and 5% PDS-CM followed by a 1 hour pulse label of ^{3}H-TDR. Magnification 1,200 x

Figure 18

Autoradiograph of primary cultures of WKY cardiocytes synchronized for 48 hours and then stimulated with 10% fetal calf serum for 16 hours followed by a 1 hour pulse of ^{3}H-TDR. Magnification 480 x

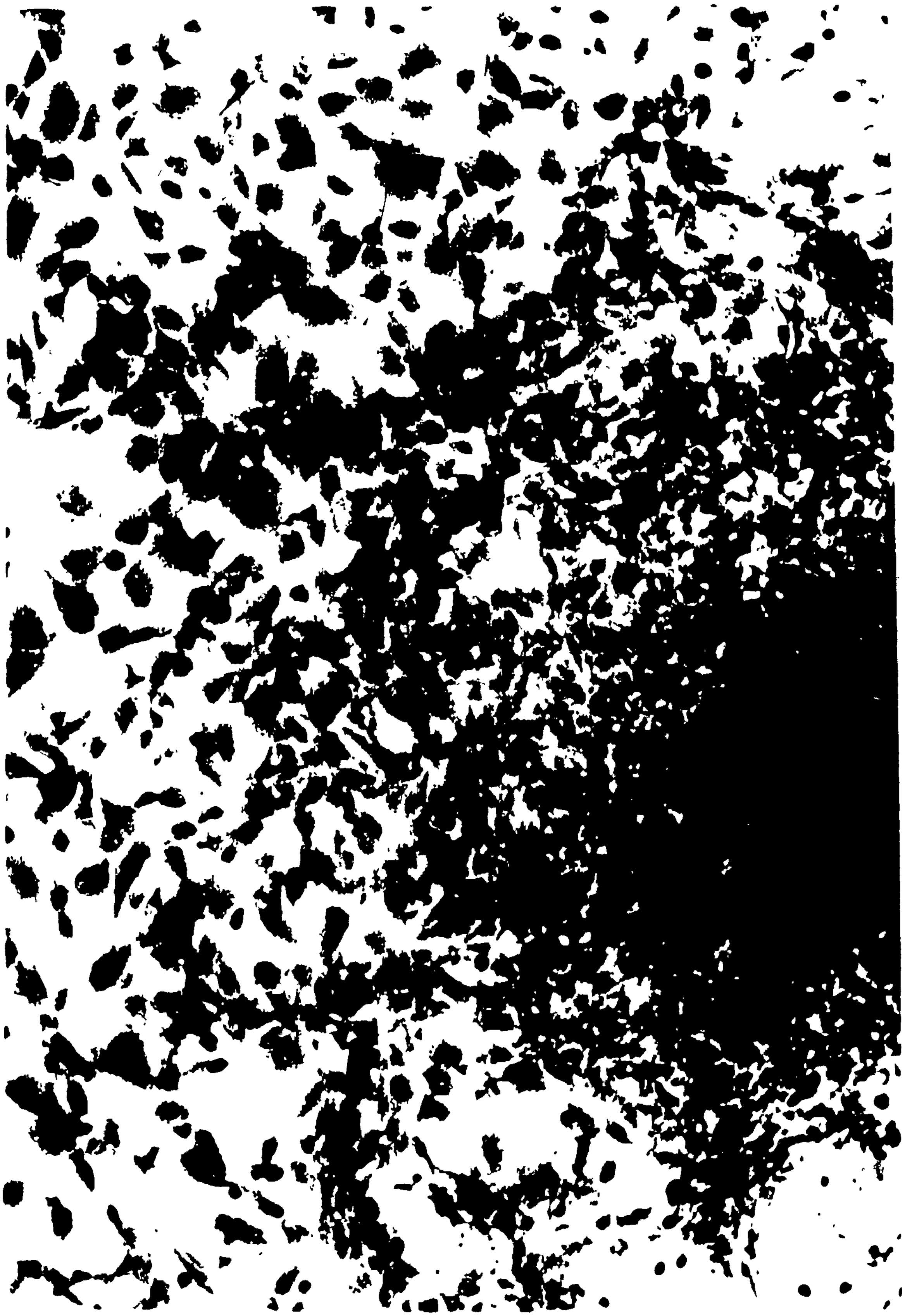


Figure 19

Autoradiograph of primary cultures of SHR cardiocytes synchronized for 48 hours and then stimulated with 10% fetal calf serum for 16 hours followed by a 1 hour pulse of ^{3}H-TDR. Magnification 480 x

vitro, and suggested that an unknown blockage of DNA synthesis occurs in culture, which masks, at least in part, the terminal differentiation of myocardiocytes.

Labelled non muscle cells were observed at both 16 and 28 hour time points providing evidence that the two peaks of DNA synthesis observed in the 36 hour time course was probably due to the shift of cells into a second cell cycle S phase. The labelling indices for asynchronous, synchronized, and stimulated cardiocytes showed similar growth rates for WKY and SHR under our culture conditions.

C - Flow Cytometry

1 -Fixation Procedures

All fixation procedures for WKY and SHR cardiocytes distorted light scatter patterns (**Figure 20**) and DNA distribution histograms (**Figure 21**). Results were similar for WKY and SHR: fixation caused cell shrinkage producing a shift in light scatter patterns to the left; 70% ethanol lysed cell membranes; paraformaldehyde produced DNA distributions most similar to unfixed cells; and fixation with glutaraldehyde prevented fluorochrome DNA staining. Based on these findings all subsequent cell sizing were performed with unfixed cells, and DNA distributions with unfixed or paraformaldehyde-fixed cells.

2 -Cell Size

Light scatter patterns of freshly dissociated cardiocytes were identical for WKY and SHR (**Figure 22**). Although a broad spectrum of cell sizes was observed, two major populations of cells were evident. The addition of standard 10μ beads to samples of cardiocytes is shown in **Figure 23**.

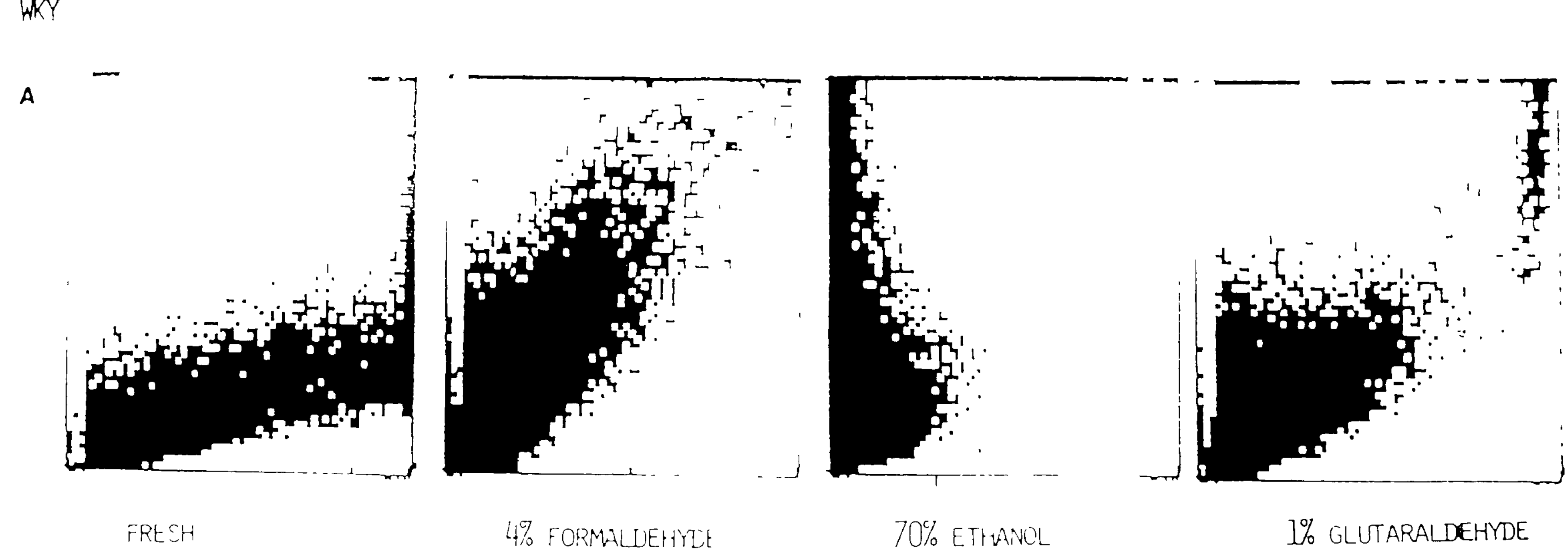
3 -DNA Distributions

a -Freshly dissociated cardiocytes

Freshly dissociated WKY and SHR cardiocytes had similar proportions of cells in G_1, S, and G_2-M phases of the cell cycle. The highest proportion of cells were in G_1 phase with only about one quarter of the cell population actively

Figure 20

Computer drawn dot plots (relative cell number vs cell size) showing the effects of various fixatives on light scatter flow cytometric analysis of freshly dissociated A. WKY and B SHR cardiocytes


WKY
A
FRESH
4% FORMALDEHYDE
70% ETHANOL
1% GLUTARALDEHYDE

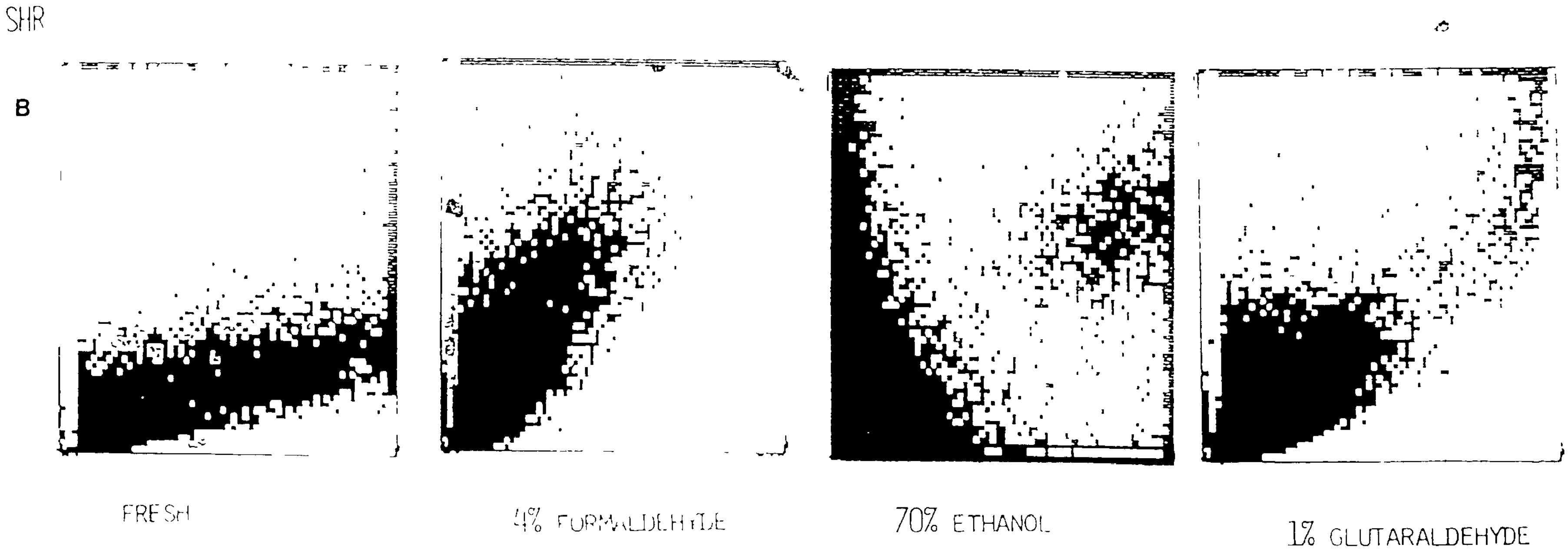

SHR
B
FRESH
4% FORMALDEHYDE
70% ETHANOL
1% GLUTARALDEHYDE

Figure 21

Computer drawn DNA distribution histograms (relative cell number vs relative DNA content) showing effects of various fixatives on flow cytometric analysis of freshly dissociated **A**. WKY and **B**. SHR cardiocytes.

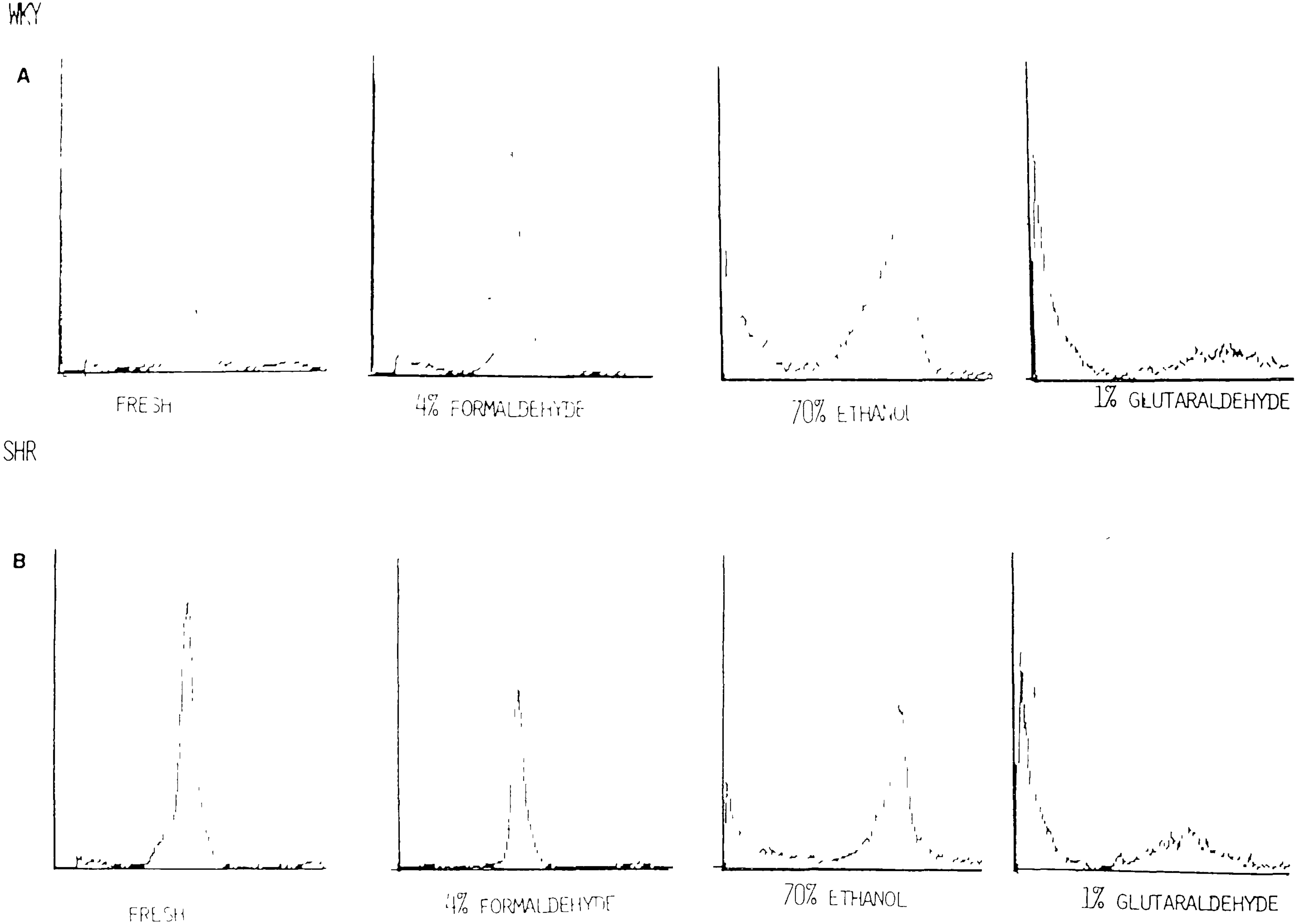

WKY
A
FRESH
4% FORMALDEHYDE
70% ETHANOL
1% GLUTARALDEHYDE
SHR
B
FRESH
4% FORMALDEHYDE
70% ETHANOL
1% GLUTARALDEHYDE

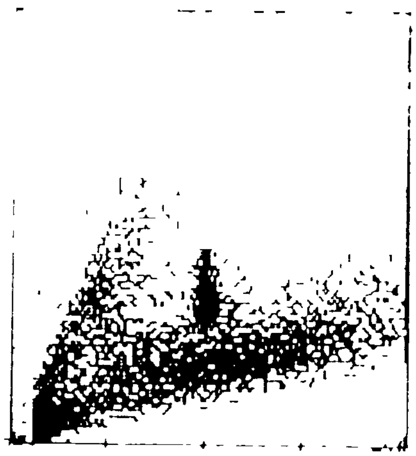
Figure 22

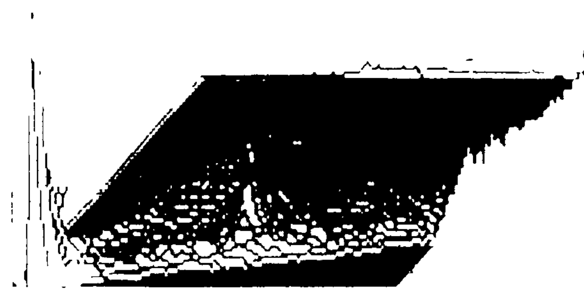
Computer drawn cell size light scatter spectrum and dot plot (relative cell number vs relative cell size) of **A.** WKY and B. SHR cardiocytes.


A
B

Figure 23

Computer drawn dot plot and three dimensional histogram (relative cell number vs relative cell size) of a sample of mixed WKY and SHR cardiocytes and standard 10 μ beads.





replicating DNA (**Table 11**). This similarity is further demonstrated in **Figure 24** where the means of 6 DNA distribution histograms each for WKY and SHR are plotted together on the same graph. In addition, comparisons of samples of WKY and SHR cardiocytes, along with a sample of WKY and SHR cardiocytes mixed together in equal proportions, further confirms the similarity (**Figure 25**); the proportion of cells in each phase for the sample of mixed cardiocytes was G_1 : 65.3 $\pm$ 3.2%, S : 25.8 $\pm$ 4.3%, and G_2-M : 8.9 $\pm$ 1.5% which was not significantly different from the WKY and SHR cardiocytes assayed independently.

b -Synchronized and stimulated cardiocytes

Cell synchronization of cultures with a 48 hr period of serum deprivation produced similar DNA distributions for WKY and SHR, shifting approximately 78% of cardiocytes into the G_1 phase of the cell cycle (**Table 12**). The time course following stimulation of cardiocytes with serum were similar for WKY and SHR (**Figure 26**); at time 0 the synchronized cells started with similar proportions of cells in each phase; 12 hours after the addition of serum a slight shift to the right was observed as some cells entered early S phase; after 14-20 hours a large proportion of the population had entered S phase which prevented accurate partitioning of cell phases; at times 24 and 28 the G_1 peak again became distinct as cells returned for a second, less synchronous cell cycle. Estimations of the proportions of

TABLE 11

ESTIMATED PROPORTIONS OF FRESHLY DISSOCIATED WKY AND SHR CARDIOCYTES IN CELL CYCLE PHASES

Strain	Cell cycle phase G_1	S	G_2-M
WKY (%) (n=10)	64.7 ± 3.5	26.6 ± 4.3	8.7 ± 1.4
SHR (%) (n=12)	65.5 ± 3.8 ns	26.5 ± 4.5 ns	8.0 ± 1.0 ns

Percentage of cells ± SE, ns - not significant

Figure 24

Computer drawn DNA distribution histogram for WKY and SHR freshly dissociated cardiocytes. Plots are means of 6 samples each

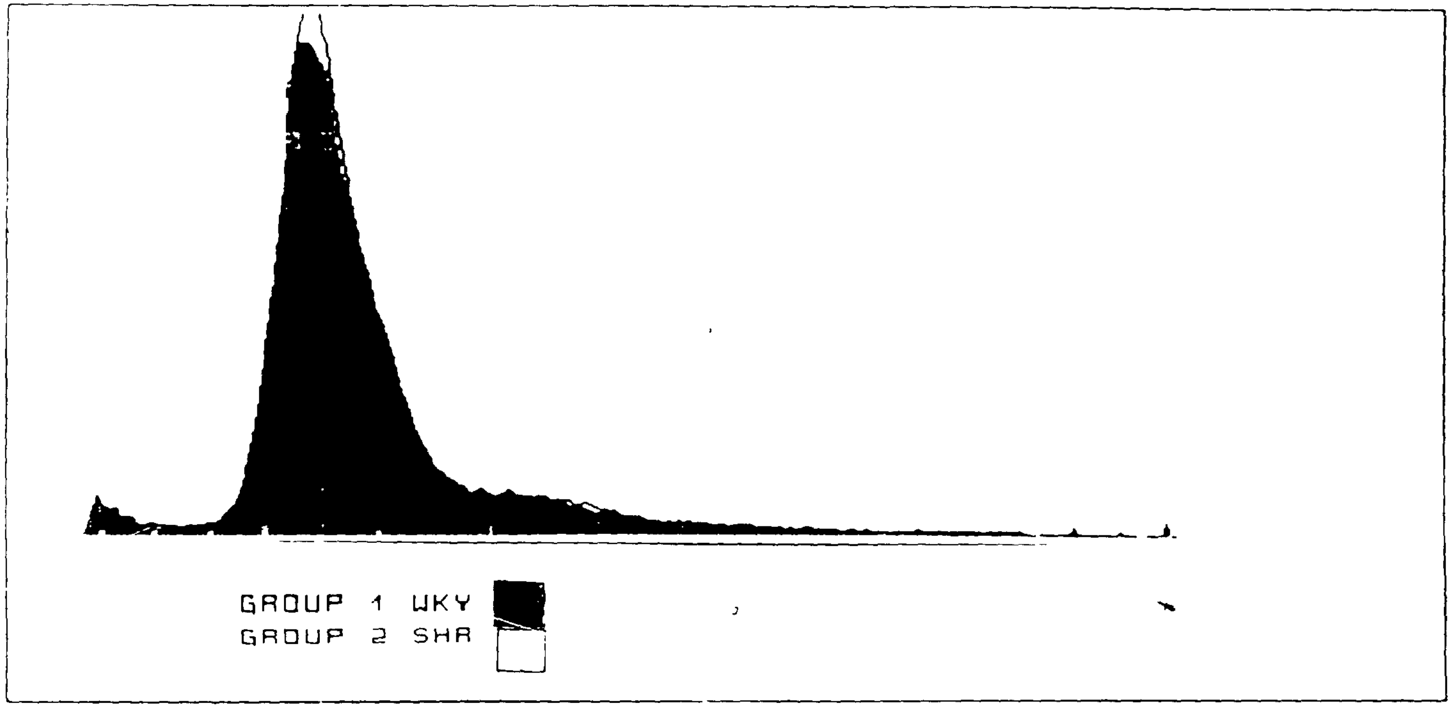

GROUP 1 WKY
GROUP 2 SHR

Figure 25

Computer drawn DNA distribution histograms (relative cell number vs relative DNA content) for freshly dissociated SHR (001), WKY (002) and mixed WKY and SHR cardiocytes (003).

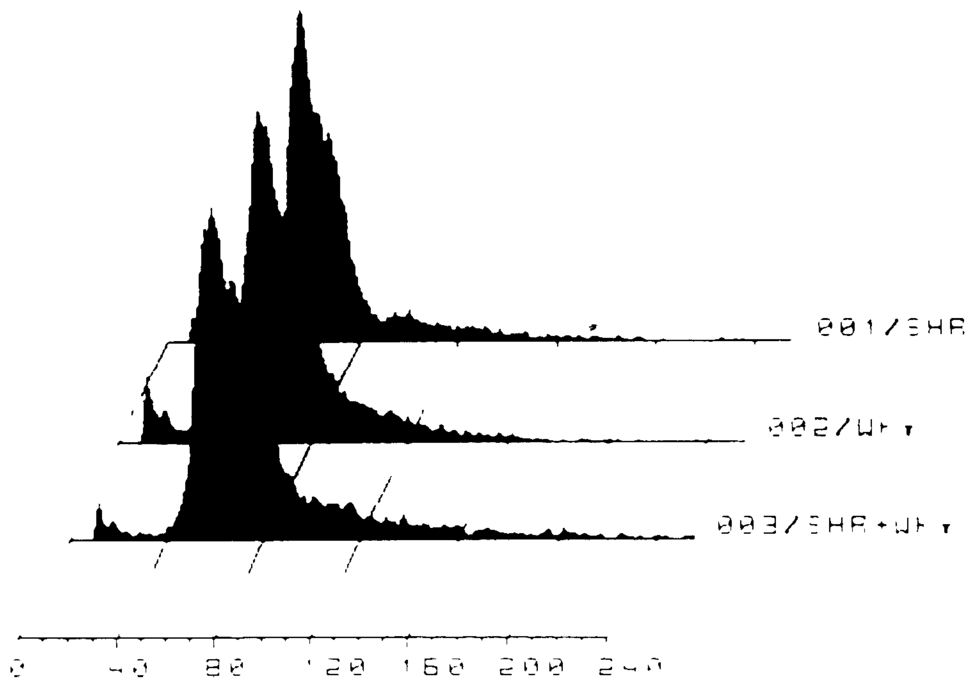

001/3HR
002/WT
003/3HR+WT
0
40
80
120
160
200
240

TABLE 12

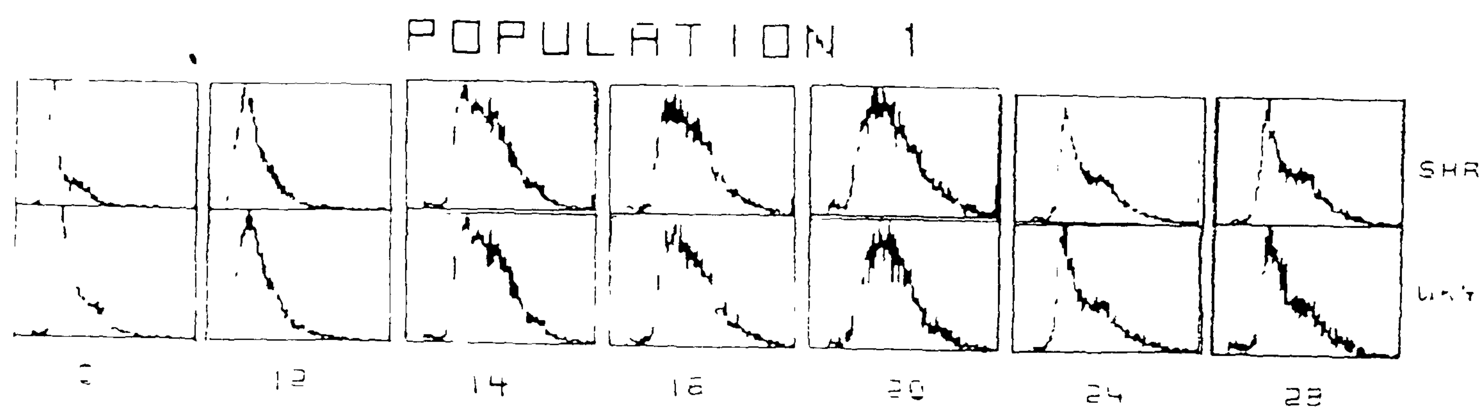
ESTIMATED PROPORTIONS OF SYNCHRONIZED WKY AND SHR CARDIOCYTES IN CELL CYCLE PHASES

Strain	Cell cycle phase G_1	S	G_2-M
WKY (%) (n=10)	78.2 ± 1.3	13.5 ± 1.0	8.3 ± 2.3
SHR (%) (n=10)	78.5 ± 1.4	14.4 ± 1.3	7.1 ± 1.2

Percentage of cells ± SE

Figure 26

Computer drawn distribution histograms (relative cell number vs relative DNA content for time course following stimulation of synchronized WKY and SHR cardiocytes with 5% FCS


POPULATION 1
SHR
12
14
16
20
24

cells in each phase at each time point did not show any significant differences between WKY and SHR (**Table 13**).

c - Subpopulations and tissue-related differences

Throughout determinations of DNA distributions the presence of an additional population (population 2) of non-cycling cells with a G_1 phase DNA content shifted to the right of the major peak (population 1)(**Figure 26**) was observed. This phenomena was present for both WKY and SHR cardiocytes (**Figure 27** and **28**) and was responsible for the relatively high coefficients of variation (9.5 $\pm$ 1.1%) obtained from both WKY and SHR cytometric readings. With cytometric gating it was possible to separate the two populations and observe them separately, thus lowering the coefficient of variation to (3.2 $\pm$ 1.0%).

Comparisons of light scatter patterns (DNA vs cell size) for newborn WKY heart, spleen, blood, and lung showed similar DNA contents for all organs with the exception of heart (**Figure 29**). The altered reading was more clearly observed in **Figure 30**, a sample of vascular smooth muscle cells from adult Wistar aorta was also included and a small shift was also observed.

4 - Discussion

Flow cytometry has several advantages over ^{3}H-TDR methods of growth determinations: cell size along with DNA per cell can be estimated; cells in G_2-M, and G_1 in addition to S phase, can be observed; variables affecting thymidine

TABLE 13

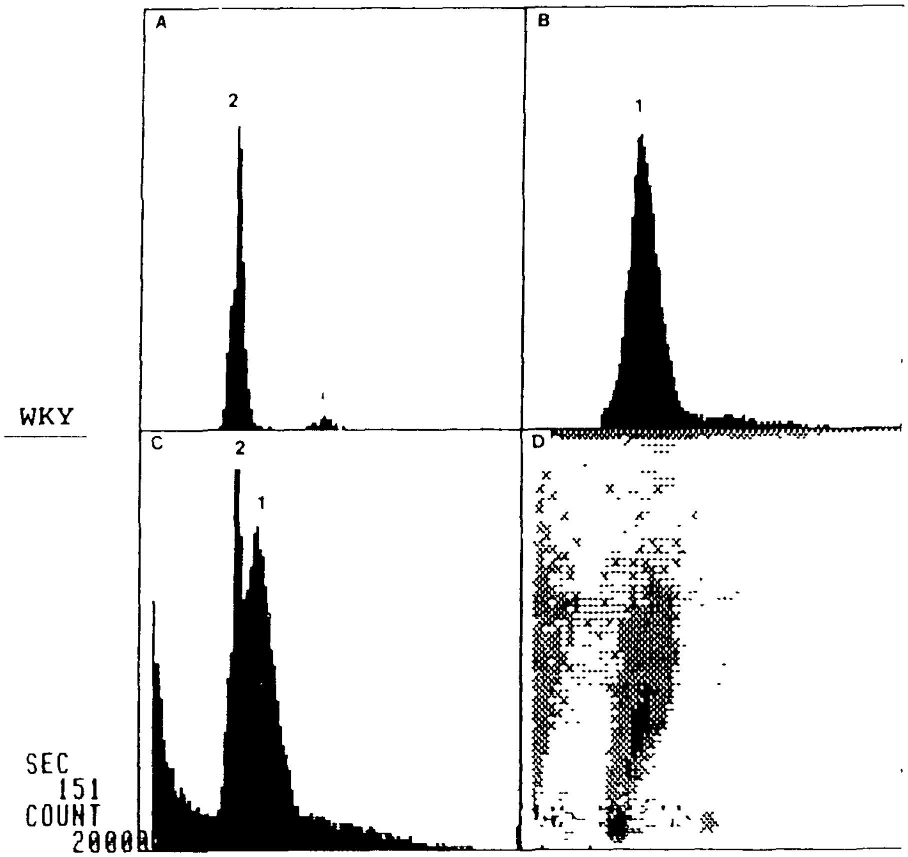
ESTIMATED PROPORTIONS OF SYNCHRONIZED WKY AND SHR CARDIOCYTES
DURING TIME COURSE FOLLOWING SERUM STIMULATION

Hours		0	12	14	16	20	24	28
G_1	WKY	78 5±1 1	73 7±1 4	52 7±4 4	39 9±3 5	32 9±4 4	58 3±3 0	54 2±3.5
	SHR	79 3±1 1	74 3±1 2	53 5±4 2	31 2±3 1	34 2±4 2	58 7±2 6	54 1±2 2
S	WKY	14 2±0 9	18 8±1 1	38 2±5 3	45 2±4 8	41 7±4 7	22 4±3 6	26 7±3 0
	SHR	13 1±0 9	19 9±1 0	37 8±5 0	44 8±4 7	41 6±4 6	21 3±3 2	27 5±2 6
G_2M	WKY	7 2±1 0	7 5±1 0	9 1±3 8	14 2±3 8	25 4±4 1	19 3±2 4	19 1±2 7
	SHR	7 4±0 6	7 8±1 0	8 7±3 6	14 0±3 8	24 2±4 0	20 0±1 9	18 4±1 6

Percentage of cells ± SE

Figure 27

Subpopulations observed for freshly dissociated WKY and SHR cardiocytes. **Panel C** - the DNA distribution histogram for total population of cardiocytes revealing the presence of two subpopulations: a major peak (population 1) and a peak shifted to the right with lower DNA content (population 2); **Panel D** - two parameter light scatter analysis (size vs DNA content); and separation of subpopulations with flow cytometric gating into population 2 (**Panel A**) and population 1 (**Panel B**).


WKY
A
2
B
1
C
2
1
D
SEC
151
COUNT
20000

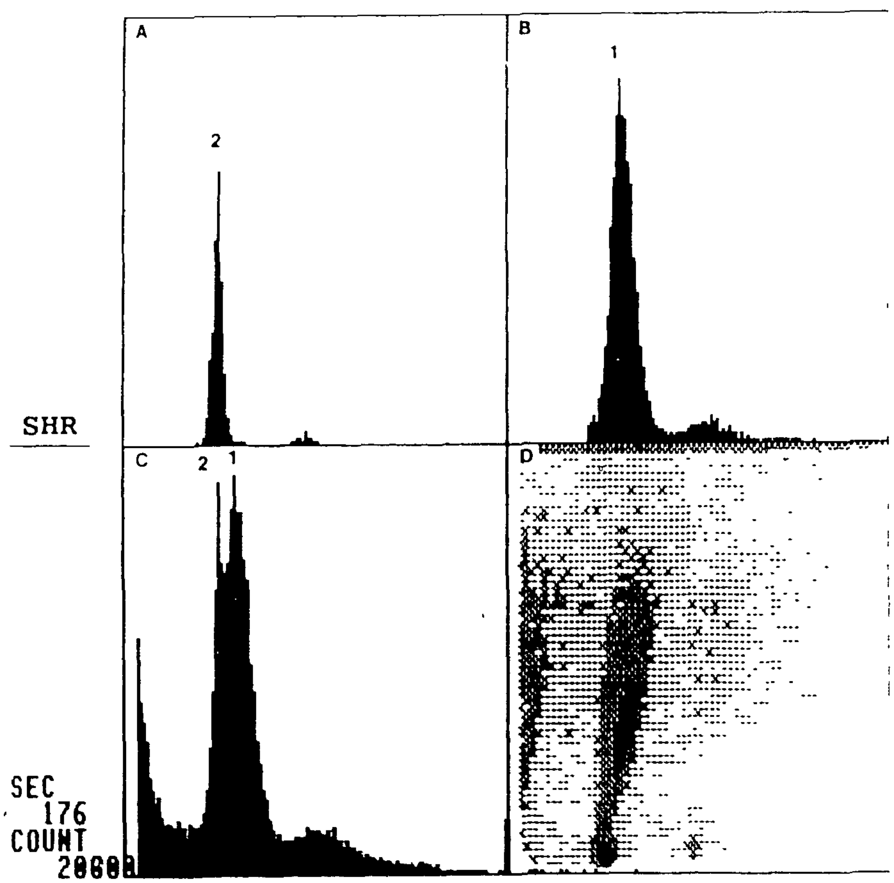

SHR
A
2
B
1
C
2
1
D
SEC
176
COUNT
20000

Figure 28

Computer drawn DNA distribution histograms (relative cell number vs relative DNA content) (population 2) following stimulation of synchronized WKY and SHR cardiocytes with 5% FCS.

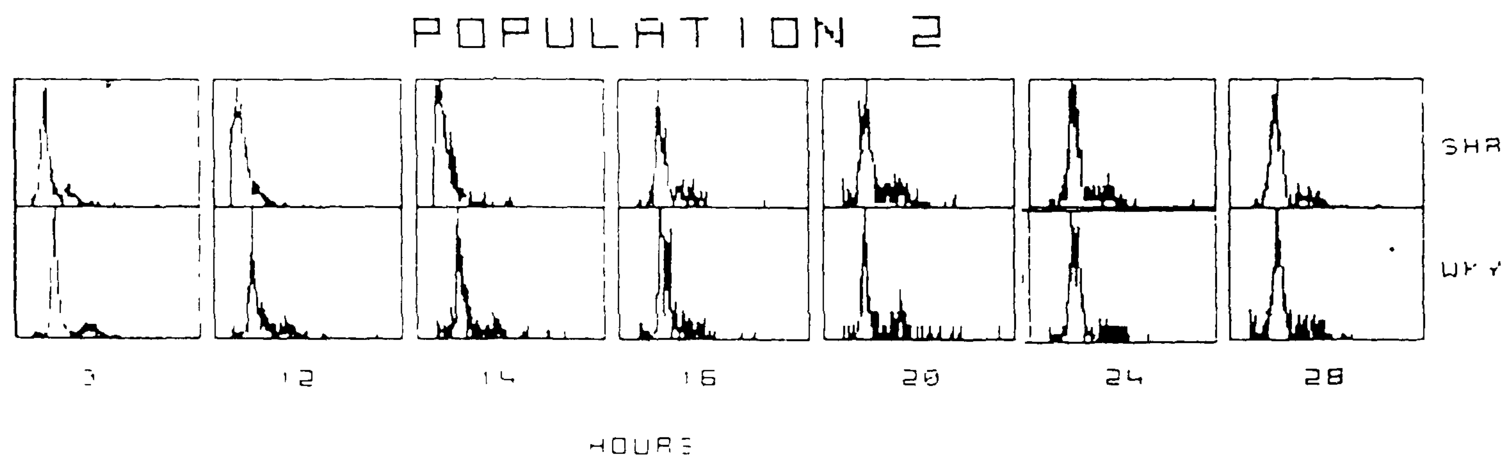

POPULATION 2
SHR
WKY
12
14
16
20
24
28
HOURS

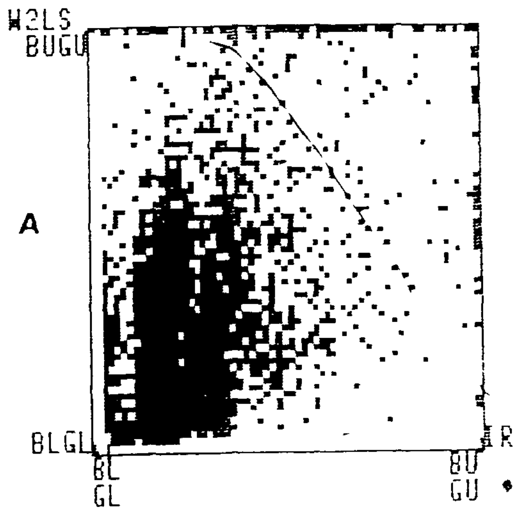
Figure 29

Two parameter light scatter analysis (DNA vs cell size) for freshly dissociated cells derived from newborn WKY A. heart, B. spleen, C blood and D. lung.

TWO PARAMETER ANALYSIS

HEART -48 25/10/85 4 31
2P64 DNA
IR -W2LS /IR
629 10000

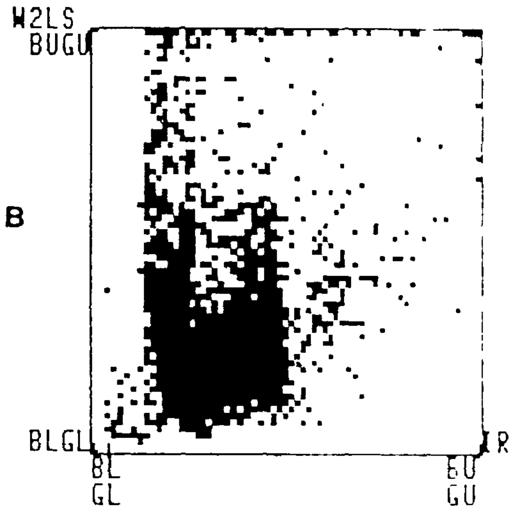
A



TWO PARAMETER ANALYSIS

SPLEEN -58 25/10/85 5 22
2P64 CALIB
IR -W2LS /IR
724 10000

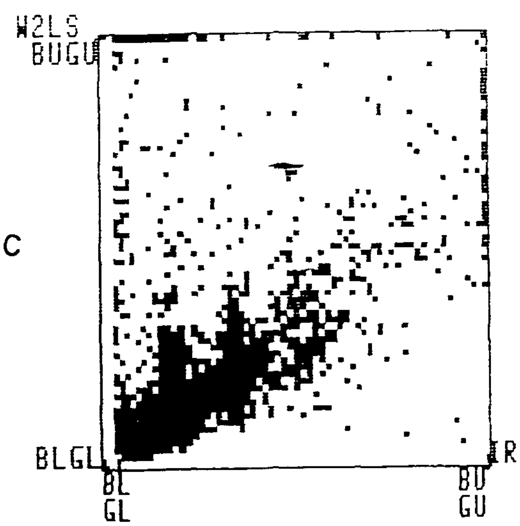
B



TWO PARAMETER ANALYSIS

BLOOD -52 25/10/85 4 45
2P64 DNA
IP -W2LS /IP
163 4560

C



TWO PARAMETER ANALYSIS

LUNG -51 25/10/85 4 43
2P64 DNA
IR -W2LS /IR
627 10000

D

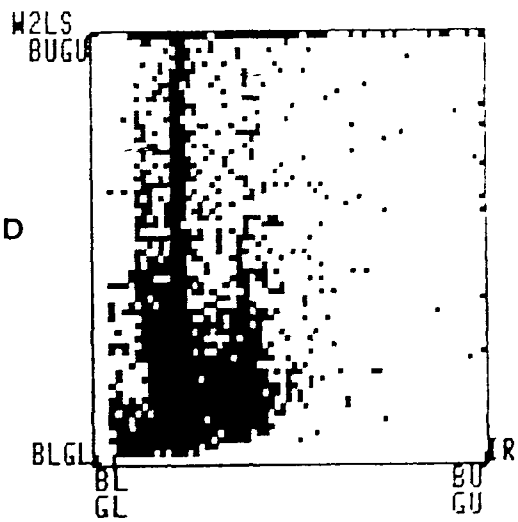
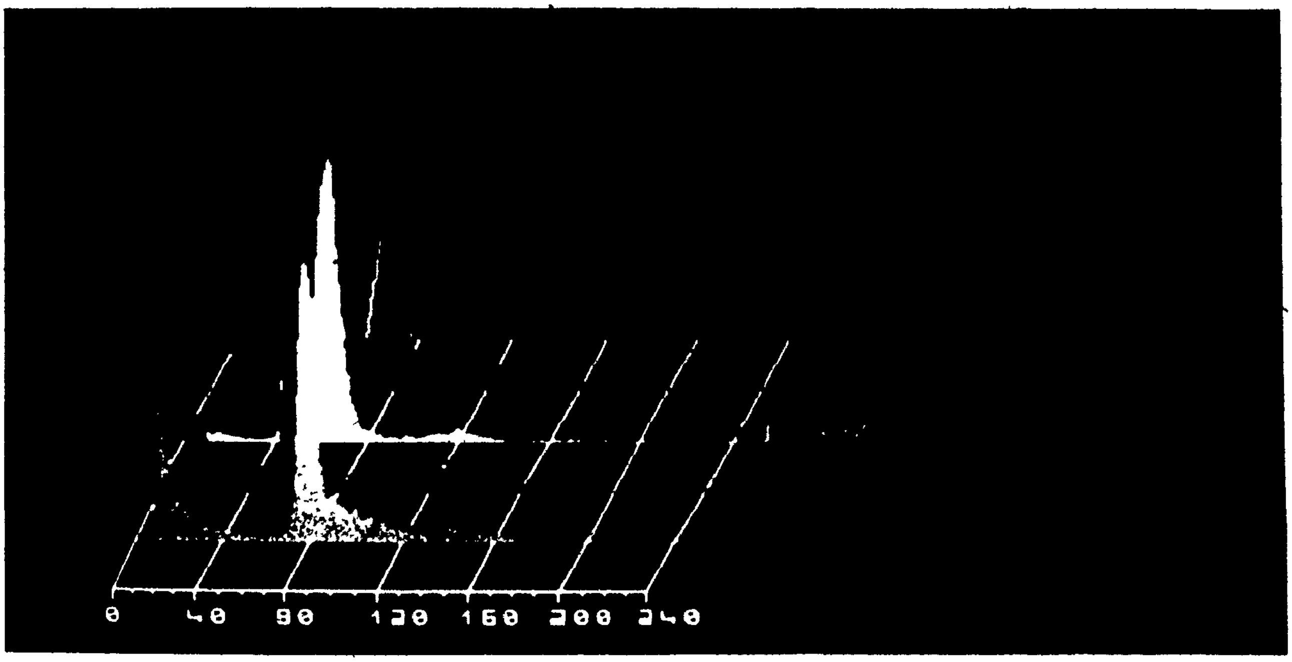


Figure 30

The DNA distribution histograms (relative cell number vs channel number) of freshly dissociated cells derived from newborn WKY spleen, heart, lung and blood, along with vascular smooth muscle cells derived from adult WKY.


0
40
80
120
160
200
240

transport and incorporation are eliminated; large and precise cell sample sizes can be analyzed quickly; and cells can be analyzed in suspension prior to culture. Despite the advances in cytokinetics provided by flow cytometric analysis several limiting factors still exist. The major difficulty is partitioning and interpreting the resulting DNA histograms. This is primarily due to the considerable overlap between the fluorescence intensities of cells in G_1 and early S phase, and between those in G_2 and M and late S phase. The overlapping fluorescence distributions result principally from instrumental fluctuations and drift, and intercellular variation in binding of fluorescent dye among cells with the same DNA content[323]. Also, as the proportion of cells in S phase increases relative to the other phases, and the G_2-M peak becomes less distinct, it becomes increasingly more difficult to uniquely partition the distribution in the three phases; as was observed for the WKY and SHR cardiocytes 14 to 20 hours after serum stimulation. The effect of abundant S cells is often further exaggerated by high cytochemical and instrumental variability[323].

Variability may also be a result of subpopulations, heterogenous with respect to their cell-cycle parameters, within a homologous cell population. For example, the existence of a G_0 phase in which cells have temporarily left the cycle and are not maturing toward division. In addition, cell death and cell decaying also contribute to population heterogeneity, cells that are reproductively or metabolically dead but still physically present in the population are decycled cells as long as they cannot be distinguished from living cells by DNA content or other criteria. Heterogeneity in the cell population may also be produced during the dispersal of solid tissue into representative suspensions of single cells. Although a variety of dispersal techniques exist for different tissue types, few have proven adequate for kinetic analysis with flow cytometry. Cell-dispersal techniques, if too harsh, may degrade some cells, leaving them with a fraction of their original DNA content, and if too mild may leave some cells joined together. The DNA distribution, particularly the G_1 peak, tends to become skewed to lower fluorescence values if the dispersal is too harsh, and a continuum that underlies the DNA distribution may be produced as well. Cell clumps affect DNA distributions by nonrandomly increasing the area of portions of the distribution. For example, a population with a relatively large G_1 fraction (the usual situation) will have clumps occurring at twice, or three times the G_1 peak location. This causes an apparent increase in the G_2-M peak and produces new peaks at the other locations. Since there are few biologic causes of triploidy, the three times peak is a good indication of clumps. Therefore, possible explanations for the presence of the second cell population observed for our WKY and SHR cardiocyte preparations include: cells in G_0; decycled or dead cells; cells degraded by trypsin dissociation; and variation in binding of fluorescent dye. A variation in binding of fluorescent dye may also be responsible for the altered DNA distribution observed for newborn WKY heart when compared to other organs. Highly significant differences in fluorescence and individual DNA differences have also been observed by Vindelov et al[323] with flow cytometric analysis of mouse liver, spleen, kidney, thymus, blood, and bone marrow. They suggested that heterogeneity of cell populations and nonstoichiometry of the dye binding were contributing factors for these differences.

With flow cytometric analysis, no significant differences in cell size and DNA distributions for WKY and SHR freshly trypsinized and cultured cardiocytes were observed. Similar cell sizes for WKY and SHR cardiocytes provided additional evidence that cardiac enlargement in SHR is not a result of cellular hypertrophy (enlarged preexisting cells) but due to hyperplasia (increased cell number). The similar DNA distribution histograms for synchronized WKY and SHR cardiocytes eliminates the possibility of altered ploidy as an explanation for the higher DNA contents of newborn SHR heart. Although it is not possible with flow cytometry to distinguish binucleated cells from mitotic cells which continue to cytokinesis, polyploidy in SHR would have increased the proportion of cells at the 2N (or 4N etc.) positions of the histogram for SHR cardiocytes. The small proportion of cells at the 2N position observed for WKY and SHR cardiocytes (approximately 8%) is within the range of percent binucleation observed by others, Clubb et al[33] observed only 4% binucleated cells in newborn rat cardiocytes, and Korecky et al[324] observed 13% binucleation.

The similar DNA distributions for freshly dissociated cells from WKY and SHR is an important observation because variables such as plating selectivity, synchronization, and serum sensitivity are eliminated. Most significantly the 26% of cells observed in S phase for both WKY and SHR did not indicate a faster proliferative state for the SHR cells; an enhanced growth rate would have been reflected by a higher proportion of cells in S phase for SHR. Once cultured, WKY and SHR cardiocytes synchronized and proceeded through the cell cycle at apparently similar rates, however, flow cytometric analysis did not prove to be sensitive enough when large proportions of cardiocytes entered S phase and an increase in proliferative rate may not be detected until our procedure is perfected further. The time course with flow cytometry was consistent with the 36 hour time course of ^{3}H-TDR incorporation into DNA.

In conclusion, *in vitro* studies showed similar DNA content per cell and cell cycle length for WKY and SHR cardiocytes. These results support the hypothesis that the higher DNA content and specific activity derived from ^{3}H-TDR observed for SHR heart *in vivo* is due to a greater number of "normally" (with respect to WKY) growing cardiocytes, with a normal proportion (or percent) of newly "born" cells. For example, if the total number of cells in SHR is 150 vs 100 in WKY, then 26% of the cells in S phase will represent a greater number of cells for SHR (27-WKY, 39-SHR) which will be reflected by an increase in ^{3}H-TDR label in SHR.

D - EXTRINSIC GROWTH STIMULI

1 - Protein Contents

The protein contents of plasma derived serum (PDS), whole blood serum (WBS), and platelet extract (PE) were not significantly different for WKY and SHR (Basic Data **Table** 14). No significant differences in protein content were observed for male and female rats and therefore, values were combined and means recorded (**Table** 15).

2 - Cardioctyes

a - Dose response to serum

A positive correlation was observed between ^{3}H-TDR incorporation into DNA and fetal calf serum and calf serum (**Table** 16 and 17) concentrations. As expected, FCS had a higher stimulating potential than CS. The cpm/cluster wells were lower for SHR cardiocytes at all serum concentrations, but was not significantly different from WKY.

b - Effect of WKY and SHR WBS and PDS on cardiocytes

Stimulation of DNA synthesis with PDS and WBS was extremely low for WKY and SHR cardioctyes **Table** 18 A and B. The rate of DNA synthesis of WKY cardiocytes calculated with 10% calf serum taken to be 100%, was 18.5% and 18.7% for WKY and SHR 10% PDS respectively. The WBS was more growth supportive with 47.0% and 44.0% stimulation with 10% WBS from WKY and SHR respectively.

TABLE 14

BASIC DATA FOR RATS USED FOR BLOOD COMPONENT PREPARATION

STRAIN	NO.	SEX	BWT	BP	PULSE
WKY	1	male	325	110 ± 2	420 ± 10
	2	male	277	120 ± 1	420 ± 10
	3	male	305	110 ± 2	420 ± 10
	4	female	168	110 ± 2	360 ± 10
	5	female	172	110 ± 2	360 ± 10
SHR	1	male	246	177 ± 3	420 ± 10
	2	male	287	175 ± 2	480 ± 10
	3	male	276	165 ± 2	560 ± 10
	4	female	164	150 ± 2	420 ± 10
	5	female	154	150 ± 2	480 ± 10

Data are means ± SE of three readings each.
BWT - body weight (g)
BP - systolic blood pressure (mmHg)
Pulse - (beats/min)

TABLE 15

PROTEIN CONTENT mg/10μl sample

	WKY (n=10)	SHR (n=10)
PDS	60.2 ± 5.4	63.0 ± 8.7$_{ns}$
WBS	83.2 ± 5.5	76.0 ± 6.8$_{ns}$
PE	2.1 ± 0.4	2.0 ± 0.6$_{ns}$

Data are means ± SE, ns-not significant

TABLE 16

DOSE RESPONSE OF FETAL CALF SERUM

ON WKY AND SHR CARDIOCYTES

FCS concentration (%)	cpm/well x 10^3 WKY	SHR
0	0.36 ± 0.03	0.34 ± 0.02
1	0.66 ± 0.05	0.59 ± 0.04
5	1.73 ± 0.23	1.66 ± 0.19
10	3.24 ± 0.22	3.20 ± 0.19
15	6.52 ± 0.27	6.43 ± 0.26
20	8.30 ± 0.20	8.29 ± 0.29

TABLE 17

DOSE RESPONSE OF CALF SERUM

ON WKY AND SHR CARDIOCYTES

CS concentration (%)	cpm/well x 10^3 WKY	SHR
0	0.40 ± 0.03	0.39 ± 0.03
1	0.52 ± 0.09	0.50 ± 0.08
5	1.25 ± 0.19	1.21 ± 0.17
10	1.97 ± 0.20	1.88 ± 0.19
15	2.73 ± 0.19	2.68 ± 0.20
20	3.85 ± 0.19	3.74 ± 0.20

Data are mean cpm ± SE; WKY n=12, SHR n=12

TABLE 18

DOSE RESPONSE OF PDS AND WBS FROM WKY AND SHR ON CARDIOCYTES

A. Stimulation of WKY cardiocytes

PDS concentration (%)	WKY cpm/well	SHR cpm/well
0	190 ± 4	191 + 3
0.5	191 ± 4	192 ± 4
2	397 ± 7	394 ± 6
5	487 ± 5	489 ± 5
10	568 ± 4	563 ± 5

WBS concentration (%)	WKY cpm/well	SHR cpm/well
0	200 ± 4	198 ± 3
0.5	510 ± 4	507 ± 3
2	687 ± 6	677 ± 7
5	813 ± 5	810 ± 6
10	1,058 ± 5	1,018 ± 6

B. Stimulation of SHR cardiocytes

PDS concentration (%)	WKY cpm/well	SHR cpm/well
0	176 ± 3	182 + 3
0.5	185 ± 4	180 ± 3
2	308 ± 6	295 ± 5
5	420 ± 6	410 ± 5
10	540 ± 5	532 ± 4

WBS concentration (%)	WKY cpm/well	SHR cpm/well
0	180 + 3	169 + 2
0.5	475 ± 3	462 ± 3
2	659 ± 5	631 ± 4
5	798 ± 6	770 ± 7
10	985 ± 5	970 ± 6

Data are mean cpm ± SE; WKY n=4, SHR n=4 c - Effect of calf serum, WBS, PDS and PE on VSMC

Higher rates of DNA synthesis were obtained with WKY vascular smooth muscle cells than newborn cardiocytes; with 10% CS over four times more incorporation of ^{3}H-TDR into DNA was observed for VSMC. Therefore, VSMC provided a more sensitive model for blood component testing. No significant differences in stimulation were observed for WKY and SHR WBS, PDS and PE (**Figure 31, Table 19**). With 10% CS taken as 100%, 55.9% and 53.8% stimulation was observed for WKY and SHR 5% PDS; and DNA stimulation with 5% WBS was 71.1% and 71.4% for WKY and SHR. The cpm/mg protein for PDS, WBS and PE showed no significant differences for WKY and SHR.

d - Discussion

The results provide evidence that the stimulatory potential of circulatory blood factors is similar for WKY and SHR. The PDS, WBS and PE derived from WKY and SHR had identical stimulatory potential on neonatal WKY cardiocytes, SHR cardiocytes, and adult WKY aortic VSMC. The VSMC had higher rates of DNA synthesis than cardiocytes and provided a more sensitive model for studying growth stimulation. The PDS caused a slight stimulation of DNA synthesis; while, as expected, WBS was more growth supportive. The PDS is serum that is deficient in platelet mitogens by removing the platelets before allowing the coagulation procedure to occur; it is capable of maintaining long-term cell viability but is incapable of initiating cell proliferation in

Figure 31

Stimulation of WKY aortic vascular smooth muscle cells (cpm per cluster well $\pm$SD) with blood components. **Panel A** - dose response of human PDS-CM; **Panel B** - dose response of calf serum; **Panel C** - stimulation with 5% PDS from adult WKY and SHR; and **Panel D** - stimulation with 5% WBS from adult WKY and SHR.

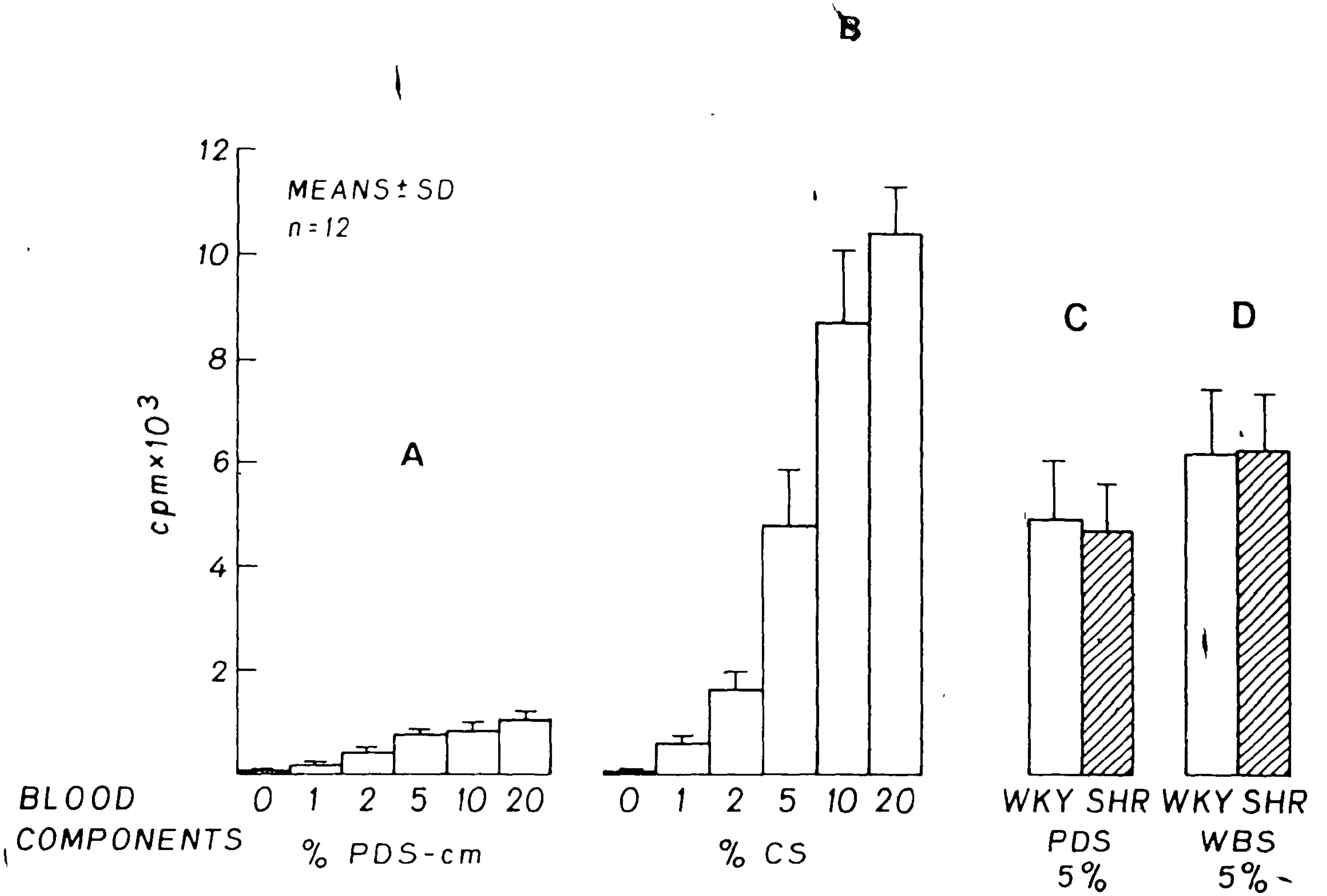

MEANS ± SD
n = 12
A
B
C
D
cpm×10³
12
10
8
6
4
2
BLOOD
COMPONENTS
0 1 2 5 10 20
% PDS-cm
0 1 2 5 10 20
% CS
WKY SHR
PDS
5%
WKY SHR
WBS
5%

TABLE 19

CPM/mg PROTEIN OF PDS, WBS AND PE STIMULATION OF VSMC

	WKY	SHR	
PDS	16.1 ± 1.2	14.7 ± 0 9	ns
WBS	14.9 ± 0.8	16.4 ± 0 7	ns
PE	25.0 ± 2 4	25.3 ± 1.6	ns

Data are mean cpm/mg protein ± SE; WKY and SHR n=18

ns - not significant.

culture. The choice of 5-10% PDS for maintenance of quiescent cultures is based upon its similarity to the milieu of cells *in vivo*. On the other hand, WBS is the fluid that results from the process of coagulation of whole blood. Thus it contains plasma constituents, plus activated coagulation and fibrinolytic factors, their respective degraded derivatives, and material released from the cellular components of the blood including platelets. Platelets contain potent mitogens such as PDGF, EGF and TGF which are released during the process of coagulation. Elements in WBS would be present only after injury associated with local platelet aggregation and release of platelet constituents. Cells exposed to WBS are therefore being exposed to a pathologic fluid.

There is evidence that PDGF may be the pathophysiologic link between the platelet and atherosclerosis, and agents that modify PDGF release and function may prove therapeutically important. Platelet extract prepared from patients with insulin-dependent diabetes mellitus caused significantly greater DNA synthesis in VSMC and fibroblasts than that of controls[325]. It was suggested that this abnormality may contribute to the vascular complication characteristic of diabetes mellitus. The present results suggest that this is not the case in high blood pressure, at least not in the SHR model.

E - BEATING RATES OF MYOCARDIOCYTES

1 -Beating Cultures

After the cells attached to the surface of the culture flask, cellular outgrowth began after 12-24 hours. The outgrowing cells were radially orientated particularly in beating colonies, while in those colonies which did not show spontaneous activity, the radial pattern of outgrowth was not found. Contracting isolated single cells were occasionally observed but even in confluent monolayer, synchronized contractions were not found. The centers of contractile activity were always the colonies (**Figure** 32). In them, even the radially outgrowing cells did not contract; but were passively displaced by the movement of the colony. However, when two neighboring colonies came into physical contact through their outgrowing cells synchronous contractions were generally observed. Beating cultures were observed up to three weeks in culture. Beating was lost following retrypsinization and replating although cells continued to grow.

a - Chronotropic effect of temperature

Tables 20 A and B show temperature effects on 5-day old and 6-day old cultures of WKY and SHR myocardiocytes. A positive chronotropic effect was observed for both WKY and SHR up to 37°C. The beating rates for SHR myocardioctyes were significantly lower than WKY at all temperatures studied.

A

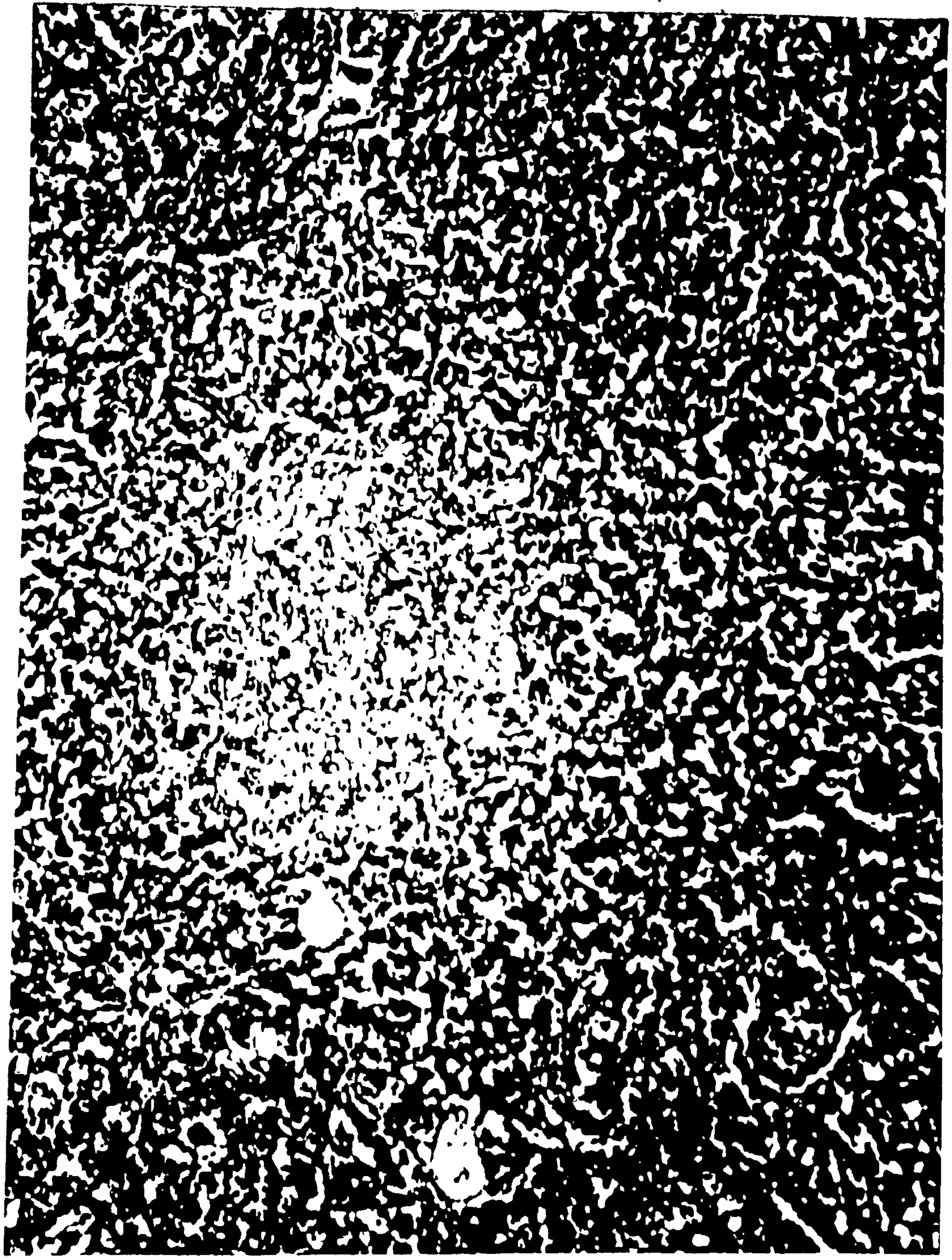


Figure 32

Photomicrograph of myocardiocyte cluster in A. relaxed and B. contracted phase. Cells in beating clusters were radially orientated with actively contracting centers, note the blurring of the cells in the cluster during contraction. Magnification 480 x

B

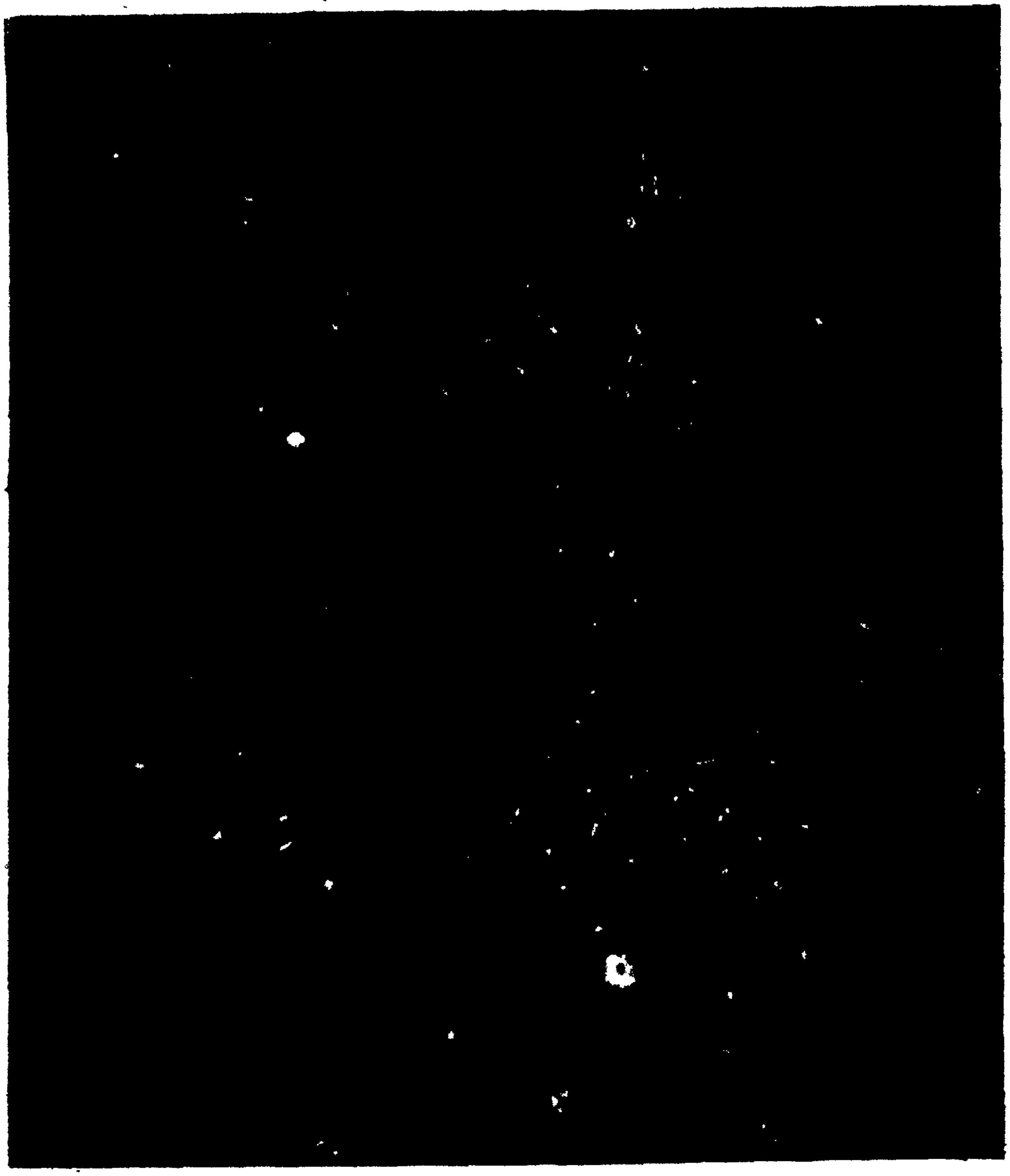


TABLE 20

CHRONOTROPIC EFFECT OF TEMPERATURE ON WKY AND SHR MYOCARDIOCYTE CULTURES

A. 5 day-old cultures

Temperature (^{o}C)	Beating rate (beats/min) WKY	SHR
27 (n=30)	85 ± 1	47 ± 1***
31 (n=30)	155 ± 3	74 ± 3***
34 (n=30)	190 ± 1	134 ± 4***
37 (n=30)	198 ± 3	185 ± 6

B. 6 day-old cultures

Temperature (^{o}C)	Beating rate (beats/min) WKY	SHR
25 (n=27)	43 ± 1	21 ± 1***
30 (n=30)	100 ± 2	64 ± 2***
34 (n=18)	126 ± 1	77 ± 1***

Data are mean beats/min ± SE
*** $p<0.001$

b - Discussion

The spontaneous beating of neonatal and fetal myocardiocytes has been the subject of many studies[326-330]. A monolayer of interconnected cells has certain advantages over the intact myocardium because the cells are separated anatomically and functionally from nerves, connective tissue, and blood vessels, and are thus used to study the direct effects of cardioactive agents without complication caused by neural and systemic influences. From these studies it has been demonstrated that cultured myocardiocytes function in a manner basically similar to the heart in situ; the rates of spontaneous beating of both single isolated myocardial cells and cell cluster change in response to changes of the potassium and calcium concentrations and to changes in temperature of the medium in essentially the same way as does the beating of adult heart.

The phenomena of spontaneous beating of myocardiocytes derived from ventricles has recently become a controversial issue[306,307,331,332]. Several investigators suggest that the spontaneous beating is actually a sign of cell damage, or "membrane leakiness", characterized by an unphysiological hypersensitivity to Ca^{+2}. This is expressed initially as a rapid and irreversible transformation of elongated, rod-like myocytes into round, highly-contracted forms which rapidly decline in viability by dye-exclusion and succinate respiration criteria. Therefore, based on this concept it is incorrect to equate beating with cell viability. It has been suggested that the cause of this hypersensitivity to calcium is a result of the dissociation procedures generally used for culture preparation. Cells are washed and dissociated in calcium-free medium to eliminate cation cohesion and therefore facilitated cell dissociation, the dissociated cells are then exposed to complete medium containing normal plasma levels of calcium. This transition causes rapid entry of Ca^{+2} in exchange for internal Na^{+2} producing Ca^{+2} sensitive cells. Several methods have been described, with varying success in decreasing beating and increasing cell viability, for preparing cultures of calcium-tolerant cells derived from adult rat ventricles. Although these methods differ in preincubation and dissociation buffers and temperatures, the common factor appears to be a preincubation period in low calcium concentrations.

This phenomena has been compared to the explosive cell death, termed "calcium paradox"[332] that is observed after perfusion of adult hearts with calcium-free solutions followed by reperfusion with solutions containing normal plasma levels of calcium. This leads to rapid cessation of contractile activity, to contracture of the heart, and to massive release of intracellular components such as creatine phosphokinase and myoglobin.

In a study designed to observe if the calcium paradox in perfused hearts is age dependent, Chizzonite et al[333] examined 3-26 day-old rats for the development of sensitivity to calcium-induced cell death. They reported that the sensitivity of the rat heart to calcium-induced cell death is absent before 7 days of age and is fully developed by 15 days. From 7 to 11 days the heart exhibits a constant but minor sensitivity to changes in extracellular calcium concentration. They speculated that the lower sensitivity of the 3 to 6 day-old myocardium to changes in extracellular calcium concentration is related to the incomplete development of parts of the surface membrane system (transverse tubules, glycocalyx, and intercalated disks), of the sarcoplasmic reticulum, and of the myofibrils in the neonatal heart. Before 7 days of age, the cardiac myocyte has an underdeveloped sarcoplasmic reticulum and surface membrane system; that is, the T tubules are rudimentary, the intercalated disks are just forming and are not interdigitated, and the sarcoplasmic recticulum is sparse and less active in uptake and release of calcium than it is in the adult. This may also account for the good survival rate of cultured myocytes derived from neonatal hearts from standard procedures (with calcium-free media and selective proteolytic enzymes) when subsequently cultured in calcium-containing medium, myocytes prepared from adult hearts do no survive when cultured in calcium containing media. Similar results were obtained by Uemura et al[334] in a study comparing the age-dependent effect of calcium-free medium on perfused rabbit heart. They showed that the calcium paradox effect is minimal in the newborn rabbit becoming similar to that of the adult at 5 weeks of age. In the adult, increased sarcolemmal permeability during Ca^{2+} repletion leads to an increased cellular calcium overload and subsequent depletion of high energy phosphate.

Due to the confusion in the literature on whether spontaneous beating cultures of myocardiocytes are acceptable models for study and the difficulty in extrapolating from the intact heart, comparisons of beating rate for WKY and SHR were stopped and two methods for preparing "calcium tolerant" cardiocytes described by Haworth et al[306] and Altschuld et al[307] were attempted.

2 - Procedure for Ca^{2+}-tolerant myocardiocytes

The methods described by Haworth et al[306] and Altschuld et al[307] were designed for adult rat heart and therefore several modifications were necessary for their application to newborn heart. Newborn hearts were perfused in petri dishes rather than the perfusion apparatus described for adult heart, and concentrations and exposure times of proteolytic enzymes were reduced to compensate for the more easily dispersed newborn cardiocytes.

The modified method of Haworth[306] was the most successful in decreasing spontaneous beating for WKY and SHR myocardiocytes cultured in complete medium (**Table 21**). Although some clusters continued to exhibit spontaneous beating, the number of clusters beating, and beating rates

TABLE 21

PERCENTAGE OF BEATING CLUSTERS AND BEATING RATE OF MYOCARDIOCYTES PREPARED BY THREE DIFFERENT DISSOCIATION PROCEDURES

Dissociation Procedure	Beating clusters (%) (n=5) WKY	Beating clusters (%) (n=5) SHR	Beating rate (Beats/min) (n=20) WKY	Beating rate (Beats/min) (n=20) SHR
Cantin[292]	100%	100%	43 ± 1	21 ± 1
Haworth[306]	25%	45%	20 ± 2	35 ± 1
Altschuld[307]	100%	100%	51 ± 2	30 ± 2

Data are mean beats/min ± SE

Percentage of beating clusters was determined from 5 flasks each.

Beating rate was determined for 4 myocardiocyte clusters/flask.

were decreased for both WKY and SHR. The percentage of clusters beating, and beating rates were significantly higher for SHR than WKY with this technique, in contrast to the lower beating rates observed for SHR myocardiocytes prepared by the methods of Altschuld[307] and Cantin[292]. The cell yield was lower with the Haworth method, approximately 1×10^6 to 1.5×10^6 cells/heart were obtained, which was about one third of the yield obtained with the Cantin procedure. Plating efficiency was also greatly reduced, cells requiring up to 72 hours to attach; confluency was not observed until 20 days of initial plating. Once trypsinized and passaged cells did not attach and grow. Growth rate determined by cell number experiments showed that approximately 45% of the WKY and SHR cardiocytes had died by day 13 in culture. The modified method of Altschuld[307] was unsuccessful in eliminating spontaneous beating but gave results similar to myocardiocytes obtained by the Cantin[292] procedure.

3 -Discussion

Attempts to obtain "calcium tolerant" myocardiocytes with the modified methods of Haworth[306] and Altschuld[307] were unsuccessful in preparing high yield, viable, nonbeating cells in the present study. Although both the number, and beating rate of myocardiocyte clusters prepared by the method of Haworth were reduced, cells were somehow damaged in the process reflected by low cell yield, plating efficiency, and growth rates, along with accelerated death rates. The low cell yield was most likely due to the presence of calcium in the cell dissociation buffer. The procedure was repeated several times with lower collagenase (10 mg, 5 mg) and trypsin (0.5 mg) contents, along with shorter dissociation steps (15 min, 10 min) however, no improvements in cell characteristics were obtained.

Cell viability, contractions, and metabolic activity of the adult myocardiocytes prepared in the Haworth[306] and Altschuld[307] studies were determined with cells in suspension, and therefore information on growth characteristics of cultured cells was not provided. It should be noted that cardiocytes derived from adult and newborn rats may respond differently to dissociation techniques.

Haworth[306] showed that the critical step in preparing calcium-resistant cardiocytes was the trypsin digestion with added calcium. They suggested that trypsin confers calcium resistance by removing membrane shreds and allowing the gap junction channels in the intact cell to close thus inhibiting spontaneous beating. This is based on the concept that the gap junction regions of isolated calcium-susceptible cells still retain shreds of the junctional regions of cells which formerly were contiguous. The unseparated gap junctions once exposed to external medium may be the site of Ca^{+2} entry, stimulating spontaneous beating[335]. This phenomena may explain why spontaneous beating was no longer observed once primary WKY and SHR cultures had been trypsinized and replated in complete medium.

Despite the difficulty in obtaining "calcium tolerant" cells, significant differences in beating rate were observed for WKY and SHR for each dissociation technique; an increased beating rate was observed following modified Haworth technique and decreased beating rates observed following modified methods of Cantin and Altschuld. It is difficult to relate the differences in beating rate observed for WKY and SHR myocardiocytes *in vitro*, to differences observed *in vivo*, however, the increased beating rate for SHR myocardiocytes dissociated with the Haworth method is consistent with the increased heart rate observed for SHR in the early postnatal period[113,124].

The differences in beating rate observed for WKY and SHR *in vitro* are most likely a reflection of the altered cell membrane described for contractile cells of the cardiovascular system in SHR. For example, an increased rate of K^+ and Cl^- turnover[336], and Na^{2+} efflux[337] has been observed in SHR vascular smooth muscle cells (VSMC). Also an increase in Ca^{2+}- Mg^{2+}-ATPase activity and a 40-60% decrease in Ca^{2+}accumulating rate was observed in aortic VSMC and myocardiocytes[338]. These defects may determine the abnormal calcium compartmentalization in VSMC showed by the kinetics of calcium influx and efflux[339]. The decrease in Ca^{2+}-binding ability of the plasma membrane is the most common manifestation of the membrane defect observed in primary hypertension. Changes in content of cyclic nucleotides in SHR[340] may also be viewed as a reflection of a membrane function defect since at least some parts of the system are membrane-bound (e.g., receptors, adenylate and guanylate cyclase, protein kinases, and phosphoprotein phosphotases). Membrane alterations have been observed in SHR at the prehypertensive stage[309] and under conditions when the development of hypertension was prevented by peripheral immunosympathectomy by means of an antiserum to NGF[308]. Therefore membrane alteration may precede hypertension, with an obvious role in developing hypercontractility and increased vascular resistance of contractile cells of the cardiovascular system in hypertension.

F - THERMOLABILITY OF CARDIOCYTES

1 - Cardiocytes in Suspension

Thermolability for suspensions of WKY and SHR cardiocytes prepared from 8-10 day-old cultures are shown in Table 22. The control percent mortality, or mortality following trypsinization at 37°C, was higher for SHR (51%), than WKY (44%), but differences did not reach statistical significance. The percent mortality for SHR cardiocytes was higher than WKY at all temperatures until a 10 min exposure at 57°C where 100% mortality was observed for both WKY and SHR. The SHR cardiocytes were significantly more heat sensitive than WKY for 25 min exposures at 40°C and 20 and 30 min exposures at 45°C.

2 - Discussion

The significantly higher heat induced mortality percentages observed for SHR cardiocytes provides possible evidence that a heat tolerance abnormality may exist in SHR at the cellular level. The alarmingly high mortality percentage observed for both WKY and SHR controls indicated that the stress of trypsinization was an additional variable to be considered. Since SHR control mortality was higher than WKY, it is possible that SHR cardiocytes experienced greater damage by the dissociation process and that this was reflected in higher heat sensitivities for these cells. In order to compare the heat sensitivity of these cells it was

TABLE 22

THERMOLABILITY OF WKY AND SHR CARDIOCYTES IN SUSPENSION

Temperature (°C)	Time (min)	WKY	SHR
37		44 ± 3	51 ± 3
40	5	36 ± 5	53 ± 6
40	15	51 ± 9	68 ± 6
40	25	45 ± 4	63 ± 2**
45	5	32 ± 8	45 ± 7
45	15	61 ± 2	70 ± 6
45	20	70 ± 6	97 ± 2***
45	30	66 ± 2	87 ± 1***
45	45	92 ± 2	96 ± 2
57	10	100	100

Data are mean percent mortality as expressed by number of cells unable to exclude vital dye ± SE; WKY and SHR n=4

**$p<0.01$, ***$p<0.001$ - significant differences between WKY and SHR compared by student's T test; three way analysis of the variance showed a significant difference between WKY and SHR ($p<0.0001$)

necessary to avoid trypsinization and observe heat sensitivities of intact cultured cells.

3 - Cardiocytes in Culture

Table 23 shows thermolability for 1 day-old primary cultures of WKY and SHR cardiocytes. A 0% mortality was observed for both WKY and SHR at 37°C and after a 5 min exposure at 40°C. The SHR cardiocytes were significantly more heat sensitive than WKY for 20 min exposures at 45°C, and after 5 min at 50°C, 55°C, and 60°C. Percent mortalities for 19 day-old were also higher for SHR cultures (**Table 24**).

4 - Heat Tolerance

Heat tolerance tests for 24 hour and 48 hour primary cultures of WKY and SHR cardiocytes are shown in **Table 25.** All 24 hour cultures had lower percent mortalities than 48 hr cultures. The SHR test flasks had a significantly higher mortality percentage than WKY for both 24 and 48 hr cultures. The WKY and SHR cultures which received an initial heat exposure at 48°C for 10 min had lower percent mortalities than the control flasks. This was also true for 24 hr WKY cultures receiving a 15 min 48°C heat exposure; but this was not observed for SHR. The additional heat exposure for 48 hr cultures did not lower mortality rates.

5 - Discussion

The observation of higher cell mortalities for SHR

TABLE 23

THERMOLABILITY OF PRIMARY 1^{o} CULTURED NEONATAL CARDIOCYTES FROM WKY AND SHR

Temperature (°C)	Time (min)	First day of culture (number = 10 each) Number of dead cells as expressed by number of cells unable to exclude vital dye (percent) WKY	SHR
37		0	0
40	5	0	0
45	5	1.9 ± 0.8	1.4 + 0.5
45	10	0.6 ± 0.4	0.6 ± 0.3
45	15	0.5 ± 0.3	2.1 ± 1.0
45	20	0.7 ± 0.4	3.0 ± 0.7*
50	5	1.7 ± 0.4	4.8 ± 1.0**
55	5	5.3 ± 1.0	22.6 ± 1.6***
60	5	68.0 ± 5.2	78.6 ± 3.3**

TABLE 24

THERMOLABILITY OF 4^{0} CULTURED NEONATAL CARDIOCYTES FROM WKY AND SHR

Temperature (°C)	Time (min)	19 day old cultures (number = 10 each) Number of dead cells as expressed by number of cells unable to exclude vital dye (percent) WKY	SHR
37		2.8 ± 0.4	3.1 ± 1.4
40	5	3.5 ± 0.4	9.7 ± 1.3
45	5	1.0 ± 0.3	5.5 ± 2.6
45	10	10.5 ± 1.4	15.1 ± 2.4*
45	15	10.2 ± 0.9	27.4 ± 3.0**
45	20	32.7 ± 3.9	61.9 ± 1.8***
45	25	62.4 ± 2.8	56.9 ± 1.6
48	5	69.5 ± 4.1	73.6 ± 3.6

Data are means + SE
*p<0.05, **p<0.01, ***p<0.001

T A B L E 25

HEAT TOLERANCE OF CULTURES OF CARDIOCYTES FROM WKY AND SHR

A. 24 hr cultures

Flasks	37°C WKY	37°C SHR		37°C 6 hrs	55°C WKY	20 min SHR
Control	0	0	-		13.7 ± 1.6	31.3 ± 2.1***
Test	0	0	48°C 10 min		5.3 ± 0.5	20.3 ± 1.6***
Control	0	0	-		16.6 ± 0.8	20.4 ± 1.6
Test	0	0	48°C 15 min		12.1 ± 1.6	25.4 ± 1.8***

B. 48 hr cultures

Flasks	37°C WKY	37°C SHR		37°C 6 hrs	55°C WKY	20 min SHR
Control	0	0	-		49.6 ± 5.0	42.2 ± 1.6
Test	0	0	48°C 10 min		56.2 ± 3.2	76.0 ± 3.0***
Control	0	0	-		45.1 ± 2.6	56.7 ± 3.5*
Test	0	0	48°C 15 min		52.5 ± 2.6	87.6 ± 2.0***

Data are mean percent mortality ± SE
*p<0.05, ***p<0.001

cardiocytes in suspension and culture is the first demonstration of altered heat sensitivity at the cellular level in a hypertensive model. Additional experiments presently being completed (Malo et al unpublished results) comparing heat sensitivities of VSMC derived from adult WKY and SHR aorta have confirmed the increased heat sensitivity in another cell type in SHR. The lower heat tolerance observed for SHR may be due to alterations in hsp induction. Initial studies from our laboratory, exposing primary cultures of WKY and SHR lymphocytes to 46°C for 5 min and then labelling the neosynthesized proteins with ^{75}Se-methionine followed by separation on SDS-PAGE electrophoresis, have already revealed an abnormal pattern of induction of 70K and 90K hsp,s in SHR[341].

The lower mortality rates observed for 24 hr WKY and SHR cultures than 48 hr cultures; along with the induction of heat tolerance observed only for 24 hr cultures, as reflected by lower mortality rates for cultures preheated at 48°C for 10 min, shows that the younger cultures exhibit higher heat tolerance. This may be due to the more recent exposure to the stress of cell dissociation by trypsinization which may induce hsp,s, providing a higher heat tolerance.

In conclusion, our results demonstrate that thermosensitivity can be observed *in vitro*, without involvement of whole body regulation. The fact that this thermosensitivity persists after several passages, clearly indicates that it is genetically encoded in these cells. Thus, the thermolability has a cellular genetic mechanism at least in SHR. Results from this study have been recently published[26].

V - GENERAL DISCUSSION

The observation by Hamet et al[1] of cardiac and renal hyperplasia in newborn SHR, reflected by increased relative organ weight and DNA content, presented the possibility that a growth defect existed very early in the hypertensive process in SHR. Determinations of relative organ weight and DNA contents for WKY and SHR organs in the present study are consistent with the initial results of Hamet; unlike the initial study, body weights of newborn SHR were heavier than WKY showing that the abnormality was present despite varying body weights of SHR. In addition, the observation of similar litter sizes for WKY and SHR, and assays of organs from newborns prior to suckling supports the hypothesis that the growth abnormality in SHR may be independent from factors affecting total body growth.

Our first approach in determining whether an enhanced DNA synthesis existed in newborn SHR heart was the *in vivo* study of ^{3}H-TDR incorporation into WKY and SHR organs. This study presented evidence of several alterations in SHR organs including aorta and adrenal, but most significant to the work in this thesis was the observation of higher specific activity of DNA in SHR heart.

The decision to compare growth rates of WKY and SHR cardiocytes in culture was based on the concept that the regulation of cardiac muscle cell proliferation *in vitro* may derive, in part, from intrinsic mechanisms that operate over several generations, even when the cells are isolated from their natural environment in the newborn heart. This is supported by work completed by Nag et al[302] who compared autoradiographs from 1 to 7 day-old cardiocyte cultures obtained from 14 and 18 day-old embryos labelled for 24 hours with ^{3}H-TDR. They showed higher labelling indices for myocardiocytes on day 1 of culture for 14 day-old rat embryo (82% of nonmuscle cells were labelled and 74% muscle cells) than day 7 in culture (45% nonmuscle and 10% muscle). The labelling indices of heart cells from 18-day embryo were lower than those of the 14-day embryo, approximately 58% muscle, 68% non muscle cells; these values declined to 5% muscle cells and 36% non-muscle cells on day 7 of culture. These findings showed that the potential for DNA synthesis and mitosis was higher in cells from the younger donor heart. Older cardiac muscle cells exhibited a decline in DNA replication more rapidly than cells from younger embryos.

The *in vitro* cardiocyte studies including: the 36 hour time course of ^{3}H-TDR incorporation; autoradiography; cell number; and flow cytometry each presented a different approach for evaluation of growth rates and provided valuable information on growth characteristics of cardiocytes derived from newborn rats. Despite their individual limitations, taken together it is clear that cardiocytes from newborn SHR do not have an enhanced growth potential *in vitro* under our culture conditions. However, their is no positive evidence that the enzymatic techniques for cell isolation provide a representative sample of the original tissue.

Our observation of similar growth rates for WKY and SHR cardiocytes *in vitro*; along with the similar mitogenic potentials of blood components from WKY and SHR adults, is not consistent with our *in vivo* observation of higher specific activity of DNA and relative DNA contents for newborn SHR heart. At this time several hypotheses can be proposed to explain this discrepancy: 1. the growth defect in SHR leading to cardiac hyperplasia may occur in utero and can no longer be detected at birth; 2. the growth stimulus responsible for the cardiac hyperplasia is no longer present once cardiocytes are withdrawn from their natural environment *in vivo*; and 3. an enhanced growth in SHR is not expressed under our culture conditions. Further studies comparing growth rates of cultured cardiocytes derived from WKY and SHR embryo, along with autoradiographic analysis of heart sections following *in vivo* incorporation of ^{3}H-TDR will be necessary to clarify these results. The latter is presently being completed but the results were not available at the time of thesis preparation.

The altered cardiocyte thermolability, plating efficiency and myocardiocyte beating rates observed for newborn SHR cardiocytes provided evidence that abnormalities in cardiocyte function exist in the earliest stage of a genetically predetermined model of hypertension. Although it was beyond the scope of this project to study the biochemical mechanisms responsible for the altered function in SHR, the work completed in this thesis provided new methodologies and direction for future studies. For example, possible alterations in induction of heat stress proteins and mRNA are presently being studied.

VI - CLAIM TO ORIGINALITY

A. Confirmed the presence of cardiac and renal hyperplasia in newborn SHR having higher total body weight than WKY. Provided evidence that this abnormality was not a result of other factors influencing total body growth such as litter size or suckling.

B. Demonstrated alterations *in vivo* of ^{3}H-TDR accumulation, incorporation into DNA, and specific activity of DNA for SHR organs; and most significantly, showed higher specific activity of DNA for newborn SHR heart and kidney.

C. Provided evidence of hypertrophy for newborn SHR aorta as reflected by higher relative weight and protein content; and possible alterations in protein and DNA contents for SHR adrenal.

D. Compared growth rates of WKY and SHR cardiocytes *in vitro* and adapted procedures of cell number, autoradiography, 36 hr time course, and flow cytometry to newborn cardiocytes. Showed similar growth rates for WKY and SHR cardiocytes, however SHR cardiocytes had a lower plating efficiency.

E. Showed similar mitogenic potentials of blood components prepared from adult WKY and SHR when applied to newborn cardiocytes and VSMC.

F. Demonstrated altered beating rates for cultured SHR myocardiocytes, and compared two methods for preparing Ca^{2+}-tolerant myocardiocytes from newborn WKY and SHR.

G. Showed higher thermolability for SHR cardiocytes in suspension and cultured on flasks suggesting a possible link between hypertension and alterations in heat tolerance.

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