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Ploidy and biochemical characteristics of liver cell nuclei in rats fed different amounts of dietary protein

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Ploidy and biochemical characteristics of liver cell nuclei in rats fed different amounts of dietary protein

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (CONT.)

LIST OF FIGURES

LIST OF FIGURES (CONT.)

LIST OF FIGURES (CONT.)

I. INTRODUCTION

Not only do adaptive changes due to insufficient dietary protein vary within different tissues of the same species, but a given change may be related qualitatively and quantitatively to the stage of ontogeny in that species and hence may vary markedly between growing and adult animals (Winick and Noble 1966). The influence of malnutrition in growing animals has been of considerable interest, and indices of cell number (total tissue DNA) and cell size (weight/DNA or protein/DNA) have been used to study tissue growth at the cellular level (Winick and Noble 1965; Winick and Noble 1966; Winick, Fish, and Rosso 1968). At a more fundamental level of organization, both a spatial and temporal compartmentalization may occur within the liver cell resulting in changes in one cell organelle but not another (Munro and Clark 1960; Dallman, Spirito, and Siimes 1974) or in changes in one cell organelle prior to another (Dallman and Manies 1973) or in a redistribution of components within an organelle (Munro, Waddington, and Begg 1965).

Although evidence now exists that inadequate dietary protein can exert effects in transcription (Wannemacher, Wannemacher, and Yatvin 1971; Henderson 1972; Andersson and

von der Decken 1975) as well as in translation (Mariani et al. 1966; Shaw and Filios 1968) during protein synthesis, the extent to which dietary protein plays a role in regulating the biochemical composition of the cell nucleus is less clear. For example, in isolated hepatic nuclei of protein-deficient animals, amounts of RNA have been reported to be unaffected (Munro, Waddington, and Begg 1965) whereas other studies have suggested that both RNA and protein per nucleus or per unit nuclear DNA may be reduced markedly (Wannemacher, Wannemacher, and Yatvin 1971; Andersson and von der Decken 1975).

Study of the problem is further complicated by heterogeneities in cell types and in nuclear classes within cell types. For example, liver contains numerically 65% parenchymal cells (Fukuda and Sibatani 1953a), and 97% of the remainder are stromal cells (Edwards and Koch 1964; Fabrikant 1968), although by volume over 90% of the tissue mass is parenchymal (Chalkley 1944). Also, a unique feature of nuclei is the state of ploidy and a particularly unique feature of hepatocyte nuclei is a well-documented transition from diploidy to tetraploidy during early growth (Carriere 1969). Thus, heterogeneity within nuclear classes may contribute to the

average compositional state observed, and some evidence for biochemical heterogeneity between nuclear classes has appeared (Johnston et al. 1968b; Bushnell, Whittle, and Potter 1969; Chanda and Dounce 1971).

The present investigations were undertaken in order to determine whether alterations in nuclear RNA and protein were temporal correlates of the response of the cell nucleus to dietary protein insufficiency and to study simultaneously the state of ploidy in that cell organelle.

Liver was chosen because it provided sufficient quantities of tissue, because existing methodology permitted the isolation of hepatocyte nuclei with acceptable yields and adequate degrees of purity, and because conflicting evidence had appeared regarding the state of nuclear ploidy which existed in liver following protein deficiency. Growing animals were chosen because they afforded a model in which dietary protein could be manipulated between an amount for optimal tissue growth and an amount sufficient only for maintenance of tissue weight. Hence, growth-associated changes could be assessed in a rapidly proliferating tissue as well as adaptive changes that occurred when growth was arrested by protein deficiency. In addition, because the

greatest rate of polyploidization was predicted to occur after weaning in the rat, this stage of development was selected for study.

The present experiments examined in whole cell homogenates and in nuclei the amounts of DNA, RNA, and protein, and the time-dependence of changes in amounts during dietary protein restriction. In addition, the activity of an enzyme associated with the nucleus, ribonuclease, was measured. Finally, hepatic ploidy was estimated by two independent methods to quantitate the influence of protein deficiency on this aspect of the cell nucleus.

These studies have characterized a major feature of hepatocyte nuclei, namely the occurrence of a state of tetraploidy in the natural ontogeny of the rat. Evidence is presented that the major effect of protein deficiency on hepatocyte nuclei is expressed as a suppression of the normal process of tetraploidization rather than in altered nuclear composition.

II. REVIEW OF LITERATURE

The major focus of this review is twofold. First, the normal patterns of polyploidization of liver parenchymal cells during growth and development are briefly summarized, and the evidence for nutritional control of the process is reviewed with emphasis on dietary protein as a determinant of the state of cellular ploidy. Second, the current knowledge concerning the biochemical composition of "normal" hepatocyte nuclei is summarized, and the existing data concerning compositional changes in hepatocyte nuclei of animals in a protein-deficient state are reviewed.

A. Ploidy States of Parenchymal Nuclei

1. Patterns in normal growth

Polyploidization is a well recognized process in liver parenchymal cells, and a detailed review of an extensive literature has appeared (Carriere 1969). Much of the historical background assessed in that review is beyond the scope of the present summary.

The meaning of polyploidization in a functional sense is still far from clear. In an operational sense, however, the overall process refers to the transition of cells

possessing the diploid content of DNA (2n), and characteristic of the somatic cells of that species, to cells possessing multiples of this amount. These multiple amounts of DNA content correspond to tetraploid (4n) and octaploid (8n) states usually. Tetraploid hepatocyte nuclei comprise the majority of polyploid cells in rat liver, and octaploid nuclei occur in relatively low frequency (Carriere 1969). Quantitatively, diploid parenchymal nuclei of the rat contain approximately 7 pg DNA (Falzone, Barrows, and Yiengst 1962).

The relative states of ploidy have been evaluated by five different techniques. Microspectrophotometry utilizing the Feulgen-DNA reaction allows measurements on individual nuclei and permits a frequency distribution of nuclei in various ploidy classes to be obtained, but the method has the disadvantage of providing data in relative rather than absolute values. Microscopic measurements of nuclear volume are based on the assumption that a doubling of nuclear DNA content is accompanied by an approximate doubling of the nuclear volume, but exceptions to a direct volume-ploidy relationship make this assay far from satisfactory (Carriere 1969). Interference microscopy before and after DNAse

digestion has been recommended by Bibbiani, Tongiani and Viola-Magni (1969). Classical chemical estimates require enumeration of nuclei either in whole homogenates or in isolated nuclear preparations and the chemical determination of DNA to obtain data in absolute amounts of DNA per nucleus. Finally, nuclei of diploid and tetraploid classes may be separated on the basis of their velocity of sedimentation (Falzone, Barrows, and Yiengst 1962; Albrecht 1968, Johnston et al., 1968a) and the relative distribution within each class assessed. The latter method has not been utilized to quantitate growth-associated tetraploidization or relative changes in protein deficiency.

The most convincing evidence for the appearance of polyploid hepatocytes during postnatal growth came from the studies of Naora (1957), Alfert and Geschwind (1958), and Nadal and Zajdela (1966a). Remarkable similarities in these studies are apparent.

Naora (1957) analyzed ploidy frequency in livers of rats of varying body weights and found values of 90-93% diploid parenchymal cells and 2-5% tetraploid cells in both 11 gram and 22-26 gram rats (prior to weaning). A rapid transition to tetraploid cells and a reciprocal decrease in

the frequency of diploid cells occurred concomitantly as body weight increased. In rats weighing 39 to 42, 47 to 59, and 67 to 81 grams, the frequency of diploid nuclei observed was 53, 36, and 13 percent, respectively, whereas the frequency of tetraploid nuclei was 42, 63, and 86 percent. Little additional change in ploidy was noted in rats weighing more than 100 grams; in animals weighing up to 395 grams, approximately 8 to 10% of the nuclei were diploid, 71 to 90% were tetraploid, and the remainder (0 to 21%) were octaploid.

Alfert and Geschwind (1958) estimated both the frequency and binuclearity of hepatocytes as a function of age. At 14 days of age, prior to weaning, approximately 90% of the cells were of the mononuclear diploid type, the remainder being equally distributed between mononuclear tetraploid and binuclear diploid cells. The earliest change observed was an increase in binucleated diploid cells (between 14 and 28 days) to approximately 30 percent of the total and a simultaneous decrease in mononucleated diploid cells occurred. Thereafter, (28 to 90 days) there was a gradual appearance of mononuclear tetraploid cells (to over 50 percent at 90 days) and further decreases in both mononuclear and binuclear

diploid cells. Beyond 114 days there was little additional change.

Nadal and Zajdela (1966a) observed similar trends. Between 3 and 8 weeks, the frequency of tetraploid cells had risen from less than 5% to over 50% and had stabilized at 38 weeks of age at about 70% of the cell population. Appreciable numbers of nuclei of higher states of ploidy (8n) were not observed in any of these studies. Thus, in several representative studies, striking similarities were noted in the pattern of the appearance of tetraploid nuclei during postnatal growth of the rat.

2. Patterns in protein deficiency

Conflicting evidence has appeared regarding the effect or lack of effect of diet on the quantity of cellular DNA in the nucleus (i.e., ploidy). The Boivin-Vendrely rule of constancy of the DNA per nucleus (Boivin, Vendrely, and Vendrely 1948) has been repeatedly challenged by numerous attempts to clarify an issue of varying controversy, viz., the influence of protein deficiency on the ploidy of the nucleus. Nevertheless, in general, a constancy of cellular DNA often has been assumed, and data frequently are expressed per unit DNA as an index of a "per cell" quantity. In order to reexamine carefully this aspect, the existing

evidence for dietary effects on hepatocyte ploidy have been reviewed, and studies from the literature have been assembled in tabular form.

Summaries of twelve different studies of dietary effects on hepatic nuclear ploidy are shown in Tables I and II. Those studies that describe the relationship between protein content of the diet and hepatocyte ploidy are indicated in Table I. Other studies that have examined restricted food intake and the effects of growth hormone are shown in Table II. Data are listed by investigators and indicate the type of study, its duration, and the experimental comparisons performed. Where stated by the authors, the initial and final body weights are indicated. The indices of assessing nuclear ploidy were dictated by the method used. For example, an index of DNA per nucleus in absolute units indicates that the conventional chemical method was used. DNA per nucleus in relative units or as percent of nuclei in different ploidy classes indicates that microspectrophometry was used. The relevant experimental data are given, and in the last column is a brief statement of the authors' conclusions of the study. Of these studies, seven evaluate the protein content of the diet, three evaluate the effects

of reduced food intake or fasting, and two studies examine the interaction of restricted food intake and growth hormone effects.

a. Studies of the effects of diets containing different amounts of dietary protein The seven studies testing the influence of the amount of dietary protein have yielded conflicting results (Table I). For example, Ely and Ross (1951) compared a stock diet (26% protein) with both a 12% protein and a protein-free diet and concluded, on the basis of chemical and microspectrophotometric measurements, that an increase in the mean DNA per nucleus accompanies the decreases in dietary protein. Lecomte and de Smul (1952) compared the effects when 19%, 10%, and 3% casein were fed to immature animals for 28 days. They also observed, by microspectrophometric techniques, an increase in the mean DNA per nucleus. Thomson and coworkers (1953) were unable to confirm these effects in older animals which had been protein depleted for 7 or 15 days; they concluded that protein deficiency had no significant influence on the average DNA content, measured chemically, of isolated liver cell nuclei.

Umana (1965) did not observe a difference in DNA per

TABLE I.
VARIOUS STUDIES OF THE EFFECTS OF PROTEIN RESTRICTION ON HEPATIC NUCLEAR PLOIDY IN RATS

Study	Duration	Experimental Comparisons	Body Weights (Grams)		Index of Nuclear Ploidy	Conclusions of the Study
			Initial	Final		
Ely and Ross (1951)	8-49 days	Stock diet			pg DNA/nucleus	
		12% casein	130-		10.2 ± 0.09	Average DNA per nucleus
		0% protein	160		11.7 ± 0.10	increased as the protein
					12.0 ± 0.08	content of the diet decreased
	15-40 days	Stock diet			DNA/nucleus (Relative units)	
		12% casein	130-		0.723 ± 0.080	
		0% protein	160		0.961 ± 0.092	
					1.090 ± 0.063	
Lecomte and de Smul (1952)	28 days	19% casein	60-65		DNA/nucleus (Relative units)	
		10% casein	60-65		582.4 ± 5.2	Mean DNA per nucleus is
		3% casein	60-65		603.9 ± 5.7	increased by feeding a
					638.6 ± 4.7	low protein diet
Thomson et al. (1953)	7-15 days	Stock diet		221	pg DNA-P/nucleus	
		0% protein x 7 days		199	0.913 ± 0.012	Protein deficiency does
		0% protein x 15 days		180	0.943 ± 0.038	not influence the DNA
					0.872 ± 0.038	per nucleus
Umana (1965)	21 days	20% casein	40	120	pg DNA/nucleus	
		5% zein	40	26	12.1 ± 0.7	Mean DNA per nucleus is
		5% corn	40	40	11.5 ± 0.7	unaffected in protein
					11.9 ± 1.8	deficiency
	21 days	20% casein	220	257	10.8 ± 2.5	Mean DNA per nucleus is
		5% zein	220	176	15.5 ± 1.6	increased in protein
		5% corn	220	211	14.1 ± 1.7	deficiency
		0% protein	220	156	15.8 ± 1.7	
Mariani, et al. (1966)	10-30 days	Control (at weaning)	49	49	pg DNA-P/nucleus 0.727 ± 0.085	Ploidy increases during the first ten days after
		25% casein x 10 days	49	87	1.074 ± 0.117	weaning and the increase
		20 days	49	116	1.160 ± 0.086	is blocked by feeding a
		30 days	49	149	1.253 ± 0.063	low protein diet
		5% casein x 10 days	49	48	0.765 ± 0.079	
		20 days	49	54	0.739 ± 0.068	
		30 days	49	60	0.862 ± 0.065	
Nadal and Zajdela (1966b)	1 month	Controls	44	112	Percent of Nuclei 2n 4n 57 43	Formation of polyploid
		Hypox.	44	58	95 5	hepatocytes is blocked by
		Hypox. + S.H.	45	77	75 25	either protein deficiency
		Protein-restricted	49	49	91 9	or hypophysis insufficiency.
		Protein-restricted + S.H.	50	44	94 6	Lack of growth resulting from protein restriction inhibits polyploid formation even if growth hormone is given by injection.
Umana (1967)	3-10 days	Control	200		pg DNA/nucleus 10.3 ± 1.6	Mean DNA per nucleus is increased in protein
		5% corn x 3 days	200		13.3 ± 1.7	deficiency
		5 days	200		15.2 ± 1.3	
		10 days	200		13.5 ± 2.9	

nucleus when weanling rats were fed 5% zein or 5% casein for 21 days, but found substantial increases when older animals were fed 0% or 5% protein for 21 days.

In contrast, Mariani et al. (1966) reported data consistent with ploidy increases at 10, 20, and 30 days in weanling control animals fed 25% casein. The increase was blocked when a low protein diet (5% casein) was fed.

Finally, the comprehensive study by Nadal and Zajdela (1966b) compared the effects of hypophysectomy and protein restriction as well as the reversibility of the effects by growth hormone replacement. The values shown in Table I for their study represent ploidy classes in both mononuclear and binuclear cells, and their data for male and female animals in each treatment group have been averaged. Their conclusion that either hypophyseal insufficiency or protein deficiency blocks the formation of polyploid hepatocytes was based on the observation that the inhibition of polyploid cell formation accompanying protein restriction was not reversed when growth hormone was given by injection.

Umana (1967) subsequently reinvestigated the rates of change in protein-deficient mature animals and found ploidy values to be elevated within three days.

Regarding protein deficiency, the results shown in Table I are obviously not clear cut and are in fact conflicting. Variations in animal age do not reconcile the differences because in weanling animals, ploidy was reported to increase (Lecomte and de Smul 1952), to be unaffected (Umana 1965), or to decrease relative to controls (Mariani et al. 1966; Fukuda and Sibatani 1953b). Similarly, in older animals, the index of nuclear ploidy was reported to be unaffected (Thomson et al. 1953) or to increase due to protein deficiency (Ely and Ross 1951; Umana 1965, 1967).

It is precisely these discrepant findings that have warranted a reinvestigation of the effect of low protein intake on nuclear ploidy in the liver.

b. Studies of the effects of food restriction and hypophysectomy The pertinent data regarding reduced food intake and growth hormone effects on hepatic nuclear ploidy are summarized in Table II, and the results of those studies are more consistent than the studies of the influence of low protein intake. Fukuda and Sibatani (1953b) showed that caloric restriction of young animals had no effect on the average DNA/nucleus when compared with controls of the same weight, but was lower than those values obtained with controls

TABLE II.
VARIOUS STUDIES OF THE EFFECTS OF REDUCED FOOD INTAKE ON HEPATIC NUCLEAR PLOIDY IN RATS

Study	Duration	Experimental Comparisons	Body Weights (Grams)		Index of Nuclear Ploidy	Conclusions of the Study
			Initial	Final		
Fukuda and Sibatani (1953b)	28 days	Calorie restricted Ad libitum controls	40	51 48-51	pg DNA/nucleus 9.26 9.14	Restricting food intake had no effect on ploidy when compared with weight matched controls
Naora (1957)	33 days	Ad libitum controls Restricted food intake	25-35 25-35	129 44	Percent of Nuclei 2n 4n 7.6 90.6 72.5 26.7	Change in ploidy is conditioned by the growth state and is independent of the age of the animal
Conrad and Bass (1957)	48 hours	Ad libitum controls 48 hour fasted	170-250		DNA-P/nucleus (mg x 10^{-10}) 8.88 ± 0.37 10.8 ± 0.55	Fasting increased the mean DNA per nucleus
		Ad libitum controls 48 hour fasted	170-250		Percent of Nuclei 2n 4n 8n 28.0 67.3 4.7 12.5 79.0 8.5	Fasting increased the relative percentage of tetraploid nuclei
Bass and Dunn (1957)	21 days	Ad libitum controls Controls, pair fed Hypophysectomized	132 127 123	273 155 139	Percent of Nuclei 2n 4n 8n 19.8 74.2 6.0 19.2 77.4 3.4 35.4 66.0 2.0	Pituitary hormones play a role in polyploid cell formation not mediated through reduced food intake
Di Stefano and Diermeier (1959)	8 days	Ad libitum controls Hypox., ad libitum Hypox. + G.H. Controls, pair-fed			Percent of Nuclei 2n 4n 37.5 64.3 69.2 30.8 45.1 54.9 42.6 57.4	Both growth hormone and food intake are involved in the ploidy shift from diploid to tetraploid

of the same age fed ad libitum. Similarly, Naora (1957) evaluated diploid and tetraploid frequency in animals initially weighing approximately 30 g and fed restricted amounts of food for 33 days. Controls, after the 33 days, exhibited a pronounced pattern of tetraploidy (90.6% of the nuclei were 4n) in contrast to those animals whose food intake was restricted; in the latter case approximately one-fourth of the nuclei were 4n.

The effects of acute fasting on nuclear ploidy were also studied (Conrad and Bass 1957). By chemical and microspectrophotometric techniques these authors observed an increase in DNA per nucleus as well as a slight increase in the frequency of tetraploid (4n) and octaploid (8n) nuclei.

The studies examining the role of food intake and pituitary hormones suggest that both of these factors may independently regulate hepatic nuclear ploidy. Bass and Dunn (1957) concluded that pituitary hormones, presumably growth hormone, play a role in polyploid cell formation. Using nearly mature animals (123 to 132 g body weight) they measured the frequency of nuclear classes in controls, hypophysectomized animals, and in controls pair fed with the hypophysectomized group. After 21 days, they observed a

reduction in tetraploid cell formation in the hypophysectomized group which they attributed to reduced amounts of circulating pituitary hormones. Since they did not observe such reductions in the pair fed group, they concluded that the effect of pituitary hormones was not secondary to reduced food intake. On the other hand, when Di Stefano and Diermeier (1959) included a hypophysectomized group given growth hormone, their data suggested that both growth hormone and food intake were involved in the ploidy shift from diploid to tetraploid.

Therefore, on the basis of the studies summarized in Table II, tetraploidization in rat hepatocytes seems to be growth dependent, and may require permissive amounts of growth hormone; prevention of growth by the limitation of food intake or pituitary ablation is accompanied by the prevention of the formation of tetraploid hepatocytes.

B. Biochemical Characteristics of Parenchymal Nuclei

The cell nucleus has been the subject of a number of reviews of general and specific nature. For example, the enzymology of the nucleus was reviewed by Siebert and Humphrey (1965) and by Ord and Stocken (1973); Munro (1970) summarized the nuclear composition and role in regulating

mammalian protein metabolism. Sufficient information has accrued to warrant a recent three volume treatise on this cell organelle (Busch, ed. 1974). The nuclear membrane itself has been the focus of recent reviews (Kasper 1974; Franke 1974) and the methods for nuclear isolation from liver cells and other tissues have been evaluted (Tata 1974).

1. Normal parenchymal nuclei

The apparent biochemical composition of nuclei can be influenced to a large degree by the method of isolation; however methodology has been improved and nuclei can be isolated reasonably free of cytoplasmic marker enzymes (Chauveau, Moule, and Rouiller 1956) and methods are now available that permit the isolation of pure nuclei in high yield. The most common procedures in current use are those of Blobel and Potter (1966) and Widnell and Tata (1964). As yet there is no consensus about the extent to which soluble constituents of the nucleus may be lost during purification and the resulting alteration in relative amounts of certain components (i.e. RNA, protein, DNA polymerase) in isolated nuclei (de Duve 1971; Bollum 1975).

The nuclei contain virtually all of the cellular DNA, except only 1.5% that is associated with the mitochondria

(Schneider and Kuff 1965). Therefore, studies on composition do not require exceedingly high yields of nuclei, and values are generally expressed relative to DNA. Whole hepatic nuclei isolated by the method of Blobel and Potter (1966) have been shown to contain, by weight relative to DNA, reproducible amounts of nuclear components. Ratios of RNA to DNA ranged from 0.14 to 0.23 (Blobel and Potter 1966; Kashnig and Kasper 1969; Tata 1974). Ratios of protein to DNA were 1.8 to 2.6 and ratios of phospholipid to DNA varied from 0.10 to 0.18 according to Kashnig and Kasper (1969) and Tata (1974). These preparations were remarkably free of cytoplasmic marker enzymes (Tata 1974). The nuclear DNA is largely localized in the chromatin fraction except for 5% associated with the nucleolus (Munro 1970).

Since only a small amount of whole cell RNA ($\sim$5%) is not of extranuclear origin, the ratios of RNA to DNA in the isolated nuclei inversely reflect the relative purity of the nuclear fractions (Blobel and Potter 1966; Munro 1970; Tata 1974). Furthermore, the small amount of nuclear RNA (relative to whole cell RNA) is distributed among all nuclear compartments including chromatin,

nucleolus, soluble space and nuclear membrane. The nuclear membrane accounts for only approximately 2% of the nuclear RNA, 10% of the nuclear protein, and negligible amounts of DNA (Kasper 1974).

2. Nuclei from protein-deficient animals

Dietary effects on the composition of the liver cell nucleus have been assessed indirectly by chemical estimates of total tissue nucleic acid; in spite of methodologic limitations in these estimates, Kosterlitz suggested in 1944 that nuclear DNA was not lost from the liver in protein deficiency. Six years later, Campbell and Kosterlitz (1950) verified this observation. Although the stability of cellular DNA during protein deficiency continues to be accepted for adult animals when cell division has ceased, effects of protein deficiency during earlier stages of development require further consideration. For example, during experimental malnutrition in rats, cell division is markedly depressed in both neonates and weanling animals (Winick and Noble 1966; Winick, Fish, and Rosso 1968; Jasper and Brasel 1974; Rosso and Winick 1975). Furthermore, the degree of depression of cell division and of reversibility during rehabilitation may be related inversely

to the stage of growth achieved prior to the onset of experimental malnutrition (Winick and Noble 1966). Although total organ DNA content reflects the number of nuclei, it does not define aspects of nuclear integrity, such as the relative amount of RNA and protein which may change due to an insufficiency in dietary protein.

Conflicting conclusions about the effect of protein deficiency on the composition of the hepatocyte nucleus have been drawn from studies of nuclear and nucleolar volumes. Although this type of evidence is indirect and admittedly weak, certain inferences have been made.

Stowell (1949) compared nuclear volumes of hepatocytes in control adult animals with those in rats fed a protein-free diet for as long as 92 days. Nuclear volumes increased 54% after three and five weeks of protein deprivation. After longer periods (54 and 92 days) nuclear sizes were closer to normal values. Furthermore, changes after 92 days were reversed rapidly (within 2½ days) when a diet high in protein was provided. When Ely and Ross (1951) fed three different amounts of dietary protein to immature animals (130 to 160 g), they observed significant decreases in average nuclear volumes as dietary protein decreased.

Mean nuclear volumes were reduced 13.6% and 35% in livers of animals fed 12% protein and protein-free diets, respectively, compared with those in livers of controls fed a 26% protein diet. Lecomte and de Smul (1952) did not confirm these findings in experiments with weanling rats maintained for 28 days on diets containing 19%, 3%, or 0% casein. They observed significant differences among animals in each dietary group, however, and concluded that nuclear volume was controlled by factors other than dietary protein.

Umana (1967) reported average nuclear volumes in adult rat liver when 5% protein from corn was fed and during subsequent repletion when 20% casein was fed. After three days on the low protein diet, average nuclear volumes were significantly larger than controls (472 u^3 vs. 445 u^3) but on day 5 were significantly smaller than controls (404 u^3 vs 445 u^3). On day 10, mean nuclear volumes were still significantly smaller than those of controls. When animals depleted for 10 days were refed 20% casein for 3, 6, and 12 days, the average nuclear volumes increased to 471, 499, and 502 u^3, respectively. These results were attributed to a redistribution of the relative frequency of nuclei within

arbitrarily defined size classes.

In summary, changes in nuclear volumes have not been characteristic of protein deficiency in all experiments reviewed and a clear relationship between nuclear volume and protein deficiency has not been demonstrated.

Direct and indirect evidence for nucleolar changes within the nucleus is relatively consistent. Initially, studies were contradictory, but evidence has been accumulated to show that morphologically the nucleolus is sensitive to changes in dietary protein. According to Lagerstedt (1949) a 4% protein diet, fed for up to 18 days, resulted in decreased nucleolar size that was less extreme than in starvation. In both cases, refeeding with adequate amounts of protein reversed the changes. Stowell (1949), observed that nucleolar volumes increased by 2, 50. 65, and 177% when a protein-free diet was fed for 21, 35, 54, and 92 days, respectively. When 30% casein diets were fed to animals depleted for 92 days, nucleolar volumes returned to control values within 2½ days. The effect of dietary protein on nucleolar size was reinvestigated by Stenram (1953).

Weanling animals were fed 0%, 3%. or 22% protein for 10 days; nucleolar diameters were measured as an estimate of nucleolar volumes. The nucleolar volume of control animals fed 22% casein averaged 2.4 u^3, whereas nucleolar volumes were significantly larger, 3.1 u^3 and 3.3 u^3, for those fed 3% casein and 0% protein respectively. Increases in nucleolar volume accompanying protein deficiency were observed in mature rats (Mortreuil and Zajdela 1956; Stenram 1958a). Furthermore, the changes were noted as early as 4 days (Mortreuil and Zajdela 1956) and persisted for 115 days in animals maintained on 4% casein (Svoboda and Higginson 1964).

Increased nucleolar size also occurred when any one of six essential amino acids was omitted separately from the diets, but the exclusion of histidine or leucine or phenylalanine alone had no effect (Stenram 1956). The increased nucleolar sizes that resulted from the feeding of 0% protein diets for 10 days was not accompanied by changes in the average number of nucleoli per nucleus (Stenram 1953, 1958b).

Utilizing interference microscopy, Stenram (1958a,b) obtained indirect measures of biochemical composition as a correlate of nucleolar enlargement. These investigations

suggested that nucleolar RNA, as a percentage of total nuclear constituents, was maintained during nucleolar enlargement induced by protein deficiency.

In a study of total nuclear RNA content in growing rats, Di Stefano and Diermeier (1959) reported that a seven day period of restricted food intake was accompanied by a reduction in nuclear RNA. Utilizing a cytophotometric assay, the nuclear RNA contents (measured by difference before and after ribonuclease digestion) were expressed in relative extinction units. Control animals were fed ad libitum and gained on average 43 grams body weight; nuclear RNA contents of this control group were normalized to 100 percent. Experimental animals were restricted in total food intake, consumed 35 to 40 percent of the amount of diet eaten by the control group, and gained on average 6 grams body weight; nuclear RNA contents were 80-83 percent of those in the control group. Cytoplasmic RNA, also estimated in this same study, was 75 to 79 percent of the amounts found in the control group.

In a limited number of studies data are available on RNA and protein in hepatocyte nuclei of animals in various stages of protein deficiency. These studies are summarized

in Table III.

Thomson and coworkers (1953) reported that values for RNA-P per nucleus were essentially unaffected by protein-deficiency in adult animals.

Munro, Waddington, and Begg (1965) took advantage of methodology not available to Stenram (1958a,b) to re-assess nuclear RNA content and its distribution within the nucleus during protein deficiency. Adult animals (180 g) were fed protein-free diets for five days, then fasted for 18 hours. These were compared with two other groups of animals (fasting and non-fasting) fed adequate levels of protein. When total RNA of whole nuclei was measured, there was no significant difference attributable to protein deficiency or to fasting. Values of 25.4, 25.8, and 26.7 ug RNA per 100 ug DNA were found for whole nuclei from livers of protein-depleted, adequate protein-fasted, and adequate protein but non-fasted groups, respectively. A significant redistribution of nuclear RNA was noted, however, when RNA was fractionated by salt extraction. The relative amount of RNA extractable by 0.1 M phosphate and 1 M sodium chloride was decreased in protein-depleted animals while the residue (i.e., nucleolar residue) was

TABLE III.
VARIOUS STUDIES OF THE EFFECT OF PROTEIN-DEFICIENCY ON HEPATIC NUCLEAR COMPOSITION

Study	Initial Weight or Age	Duration (Days)	Experimental Comparisons	RNA	Protein	Method of Nuclei Isolation
Thomson, et al. (1953)	200g	15	Controls 0% protein	0.180[a] 0.183		Citric acid[d]
Munro, Waddington, and Begg (1965)	180g	5	Controls (fed) controls (fasting) 0% protein (fasting)	0.267[b] 0.258 0.254		Widnell and Tata[e]
Wannemacher, Wannemacher, and Yatvin (1971)	23 days	18	18% casein 6% casein	4.17[c] 2.72	39.9[c] 19.6	Blobel and Potter[f]
Andersson and von der Decken (1975)	70-80g	6	20% egg albumin 3% egg albumin		24.6[b] 11.1	Blobel and Potter[f]

[a]pg RNA-P/nucleus

[b]mg/mg DNA

[c]pg/nucleus

[d]Mirsky and Pollister (1946)

[e]Widnell and Tata (1964)

[f]Blobel and Potter (1966)

increased. Such differences due to diet were not demonstrable in various sedimentation classes of RNA (e.g., 4 to 8 S, 18 S, 28 S, and > 35 S), and a redistribution based on these criteria did not occur.

Wannemacher et al. (1971) reported values of total nuclear RNA measured in livers of weanling rats after feeding 6% or 18% casein for 18 days. On a per nucleus basis, a 35% reduction in nuclear RNA was associated with the low protein diet (2.72 vs. 4.7 pg RNA per nucleus). Total protein was reported in the same study to decrease 50% in protein deficiency when expressed on a per cell basis. The values were 19.6 and 39.9 pg nuclear protein per cell for groups fed 6% and 18% casein, respectively.

Andersson and von der Decken (1975) also noted highly significant differences in nuclear protein content relative to DNA following 6 days of feeding 3 or 20% egg albumin. The DNA/protein ratios of the nuclear fractions were 0.0406 and 0.0903, respectively, for livers from protein-fed and protein-restricted rats. Expressed as protein/DNA, the values were 24.6 and 11.1, considerably above typical values reported by others using the same method of nuclear isolation (Tata 1974).

Presently, no studies are available in the literature describing the effects of protein nutrition on nuclear ribonuclease. The relative proportion of hepatic ribonuclease in the nuclear fraction is small according to Rosso, Nelson, and Winick (1973), and changes in activity of that fraction have accompanied postnatal growth (Bresnick, Sage, and Lanclos 1966). Thus, evaluation of the activity of this enzyme when growth was arrested by protein restriction was included in the present study.

In summary, the focus of this review has been to compare two general features of hepatic parenchymal nuclei from normal animals with those that were protein deficient. One feature, that of well-defined patterns of polyploidization during postnatal growth, is well established, but the evidence that dietary protein affected changes in those ploidy patterns is conflicting. The second feature was the "average" biochemical composition of hepatic nuclei with respect to RNA and protein. The evidence by some workers that nuclear RNA and protein changed in protein-depleted animals while others observed no changes is also conflicting in published data. The present studies suggest

that in growing animals an alteration in the ploidy pattern occurs as a physiological change; thus any compositional changes expressed on a "per nucleus" basis may reflect differences in ploidy states in addition to composition per cell.

III. MATERIALS AND METHODS

The results of these studies were obtained from three separate experiments. Some variations in design and methodology were employed.

A. Experimental Animals and Diets

Female weanling rats of the Wistar strain were either the progeny of the colony maintained by the Nutrition Laboratory at Iowa State University or were obtained commercially from Bio-Lab Corporation (formerly Simonsen Laboratories), White Bear Lake, Minnesota. Animals were fed ad libitum diets of equal caloric density containing either 5 or 20 percent casein for 4, 8, 12, or 16 days, and were deprived of food for 18 hours before they were killed.

The 20 percent casein diet contained, in g. percent: cornstarch 64, casein 20, non-nutritive fiber 2, Hawk and Oser Salt Mixture 4, and corn oil 10. Vitamins were incorporated into the diet by diluting them with 50 g of cornstarch before adding them to the other ingredients. The 5 percent casein diet was identical except that cornstarch was substituted isocalorically for casein. These two diets were prepared in 10 kilogram quantities without the addition

of vitamins, i.e. 5 g. percent cornstarch was omitted from the final mixture. At weekly intervals 50 g of a vitamin-cornstarch mixture was added to 950 g of the premix to provide 1 kilogram quantities of the final diet with: thiamine hydrochloride 1.88 mg, riboflavin 3.75 mg, pyridoxine hydrochloride 1.8 mg, niacin 22.5 mg, calcium pantothenate 12 mg, vitamin B_{12} 7.5 ug, biotin 0.3 mg, folic acid 1.5 mg, p-aminobenzoic acid 30 mg, menadione 0.15 mg, choline 1.25 g, l-ascorbic acid 75 mg, inositol 0.30 g, vitamin A palmitate 3000 IU, vitamin D_2 3000 IU, and dl-α-tocopherol, 90 mg. The additions of vitamin B_{12}, biotin, and vitamin D_2 were facilitated by first triturating each in a 100-fold or 1000-fold excess of cornstarch prior to addition to the vitamin-cornstarch mixture.

All constituents of the diet were obtained from General Biochemicals, Chagrin Falls, Ohio except that riboflavin, niacin, vitamin D_2, and dl-α-tocopherol were from Nutritional Biochemicals, and vitamin A palmitate beadlets were obtained from Sigma Chemical Company, St. Louis, Missouri.

B. Preparation of Tissue and Isolation of Nuclei

Need to improve procedures in the preparation of tissue homogenates and in the isolation of nuclei was indicated

during the course of this work. Therefore, procedures employed are described separately for each experiment. In all cases animals were deprived of food for 18 hours before they were killed. After livers were removed, all subsequent steps in the isolation procedures were performed at 0-4 C.

1. Experiment I

Livers were washed twice in 0.15 M NaCl, blotted and weighed rapidly. Nuclei were isolated according to the method of Chauveau et al. (1956). Livers were minced and transferred to a glass homogenizer tube equipped with a pestle (TRI-R Instruments, Inc., Rockville Centre, New York) having a clearance of 0.006 to 0.009 in. Clearance is defined as the difference between outside diameter of the pestle and inside diameter of the homogenizer tube. Nine volumes of 2.2 M sucrose-3mM $CaCl_2$ were added, and homogenizations were accomplished by 5 vertical strokes of the pestle (motor driven @ 1700 rpm). To remove connective tissue homogenates were filtered by passage over a column (0.5 x 8 cm) of 1/16 in. glass helices. An aliquot of each homogenate was stored at -20C; the remainder was transferred to 15 ml polycarbonate tubes and centrifuged at 20,000 rpm (40 000 x g) for 1 hour (SE-12 rotor, Sorvall RC2-B

refrigerated centrifuge). The supernatant was decanted, and the walls of the tubes and surface of the pellet were gently rinsed with cold 0.25 M sucrose - 3 mM $CaCl_2$. The nuclear pellet was then resuspended by homogenization (3 strokes @ 1700 rpm), transferred to a 15 ml polyethylene tube, rapidly frozen by immersing the tube in liquid nitrogen and stored at -20 C.

2. Experiment II

The procedure of Blobel and Potter (1966) was adapted for larger volumes (Kashnig and Kasper 1969) and used with further slight modifications. Experimental livers were washed in 4 changes of 0.25 M sucrose in TKM buffer (0.05 M Tris-HCl, 0.005 M $MgCl_2$, 0.025 M KCl, pH 7.5). After livers were blotted and weighed, they were minced in two volumes of 0.25 M sucrose in TKM buffer and transferred to a precision homogenizer tube equipped with a teflon pestle having a clearance of 0.010 in ± 0.0002 (Glenco Scientific, Houston, Tex). Homogenization was accomplished by 15 vertical strokes of the pestle (motor driven @ 1700 rpm). The resulting homogenate was filtered through two layers of grade #50 cheesecloth (28 x 24 threads/$in.^2$) having an effective filtration area of approximately 5 cm^2. Filtration was effected

by applying air pressure with a small aquarium pump. An aliquot of each filtered homogenate was stored at -20 C. Another aliquot (10 ml) of each filtrate was transferred to a 50 ml thin wall polypropylene tube and two volumes (20 ml) of 2.3 M sucrose in TKM were added by syringe and the contents were mixed with the filtrate by inversion. The resulting mixture was underlaid with 10 ml of 2.3 M sucrose in TKM buffer using a syringe equipped with a blunt needle. The layers were centrifuged for 2 hours at 13,000 rpm (18,000 x g, average) using the HB-4 swinging bucket rotor of a Sorvall RC2-B refrigerated centrifuge. The supernatant was discarded and the tube wall was wiped dry with tissue paper wrapped around a spatula. The pellet was suspended in 10 ml 0.25 M sucrose in TKM buffer. An aliquot was removed for nuclear counting by hemocytometer, and the remainder was stored at -20 C.

3. Experiment III

The basic procedure described for experiment II was used. Modifications were made only in the technique of homogenate filtration and in the use of an ultracentrifuge to permit higher g forces. Homogenates were filtered by passage over two grades of cheesecloth: the first consisted

of two layers of grade #80 (40 x 32 threads/in.^{2}), and the second filter was four layers of grade #120 (72 x 56 threads/in.^{2}). Aliquots of both crude and filtered homogenates were stored at -20 C for up to three months. An aliquot (10 ml) of each filtered homogenate was transferred to 60 ml cellulose nitrate tube, made 1.62 M with respect to sucrose in TKM buffer, and underlaid with 15 ml 2.3 M sucrose in TKM buffer as described above. The tubes were then completely filled by layering 15 ml 0.25 M sucrose in TKM buffer above the top layer in order to prevent tube collapse during centrifugation at 24,000 rpm (70,000 x g, average) for 1 hour in the SW 25.2 rotor of a Spinco L-2 ultracentrifuge. The nuclear pellets were stored at 0 C for at least 12 hours and then resuspended in 10 ml 0.25 M sucrose in TKM buffer for sucrose density gradient analyses. Storage time under these conditions had no demonstrable effect on the separation of ploidy classes by gradient centrifugation. However, to obviate possible effects of variable storage times on apparent ploidy distribution, nuclear preparations from both dietary treatment groups were isolated and analyzed by density gradient centrifugation at the same time.

Aggregation or clumping of nuclei was minimized and uniform suspensions obtained by first adding 2 to 3 drops of the suspension medium to the pellets, stirring to form a smooth paste, then adding the remainder of the 10 ml suspension medium. The suspended nuclear samples were divided into two equal portions. One portion was stored at -20 C, but was never used for further study. Another portion was stored in ice for subsequent sucrose gradient analyses.

C. Cheesecloth Filtration of Liver Homogenates

The filtration of tissue homogenates over gauze or cheesecloth is a routine procedure in most laboratories. Its purpose is to effect the selective removal of grossly visible pieces of fibrous and connective tissue and result in the preparation of a filtrate sufficiently uniform to enable precise sampling of small aliquots. However, in many cases investigators have failed to specify either the number of layers or the mesh sizes.

During the studies of experiment II significant amounts of grossly visible particles were present in homogenate filtrates when surgical grade cloth was used and these were potential contaminants of the purified nuclear fraction.

Surgical grade cloth (e.g., grade #50) having 28 x 24 threads/in.^{2} is probably the most commonly used porosity of cloth for the purpose and was used routinely in experiment II. It seemed desirable to compare the relative effectiveness of finer mesh sizes both qualitatively and quantitatively as a filter for crude hepatic homogenates.

First, two grades of cloth having relative porosities less than that of grade #50 were compared. The only objective criterion was measurement of the retention of solid materials retained on the filter after it was dried to constant weight. As shown in Table IV, when equivalent amounts of homogenate (20 ml corresponding to 6.7 grams of liver) were filtered over grade #50 cloth, retention increased with surface area. When surface area was maintained constant, the retention of solids increased to a maximum and was not increased by utilizing additional layers. Moreover, when sequential filtration was performed over 2 layers of grade #80 and then over 4 layers of grade #120, the bulk of fibrous tissue was trapped by the first filtration step and the total retained in the two steps was similar to that retained when grade #120 was used alone. When this last method was tested further, the removal of solids was directly

Table IV. Retention of liver homogenate particulate material by cheesecloth filters of different grades and surface areas

A 33 % w/v liver homogenate was prepared as described in MATERIALS AND METHODS and 20 ml were applied in duplicate to preweighed filters of varying mesh size, thickness, and surface area. After filtration under pressure (50 cm H_2O)[a], the filters were dried at 54 C to constant weight and the retention of solids determined.

Method	Grade of Cloth	Porosity (Threads/in^2)	Surface Area (cm^2)	Layers of Cloth	Retention[b] (grams)
1	50	28 x 24	5	2	0.15; 0.16
2	50	28 x 24	20	2	0.26; 0.27
3	80	40 x 32	20	2	0.33; 0.30
4	120	72 x 56	20	2	0.40; 0.42
5	120	72 x 56	20	4	0.39; 0.44
6[c]	80	40 x 32	20	2	0.33
	120	72 x 56	20	4	0.08

[a]Considerably greater filtration pressure required due to occluded flow in methods 1 and 5.

[b]Corrected for solids in the homogenizing medium.

[c]Sequential filtration.

proportional to the amount of homogenate applied over the range 5 to 20 ml (Results not shown). It was therefore concluded that if both surface area and homogenate volume were kept constant, filtration under these controlled conditions would result in reproducible filtrates with negligible differences in composition.

Finally, the effect of homogenate filtration on the apparent nucleic acid concentrations was examined. In Table V are summarized the results of two studies in which DNA and RNA were measured on aliquots of both crude (unfiltered) and filtered homogenates. Approximately 20% of the total DNA was removed by filtration and 10% of the total RNA (8 and 13% in 2 experiments). The resulting RNA/DNA ratios increased accordingly in the filtrate. These results are compatible with the selective removal of fibrous and connective tissue elements that have lower RNA/DNA ratios than that of the parenchymal cells, although they do not exclude the possibility that an unknown and variable proportion of hepatocyte cells were also retained.

The usefulness of filtration, particularly in studies involving the isolation of nuclei is represented by the data in Table VI. Since homogenate DNA concentration was lowered

Table V. Levels of DNA and RNA in crude and filtered liver homogenates

Rat liver homogenates were prepared from female Wistar rats weighing 200-300 grams. Twenty ml were sequentially filtered as described in MATERIALS AND METHODS for experiment III. Aliquots of the crude homogenates and the filtrates were analyzed for RNA and DNA and expressed as mg/gram of liver, assuming one ml of homogenate or filtrate to be equivalent to 330 mg of wet liver weight.

Experiment	Component Measured	mg/g wet weight or mg/mg		Percent Change in Filtrate Relative to Crude Homogenate
		Crude	Filtrate	
	DNA	3.11	2.50	-20
A.	RNA	8.90	7.71	-13
	RNA/DNA	2.86	3.08	+ 8
	DNA	3.19	2.52	-21
B.	RNA	8.77	8.10	- 8
	RNA/DNA	2.75	3.21	+17

Table VI. Recoveries of nuclei from crude and filtered liver homogenates

Rat liver homogenates were prepared from female Wistar rats weighing 200-300 grams and filtered as described in MATERIALS AND METHODS for experiment III. Ten ml of the crude homogenates and corresponding filtrates were used for the isolation of nuclei.

Experiment	Type of Homogenate	DNA, mg		Recovery[a]
		Homogenate	Nuclei	
A.	Crude	10.3	4.09	40
	Filtered	8.25	5.00	61
B.	Crude	10.53	4.42	42
	Filtered	8.32	4.82	58

[a]Percentage of homogenate DNA recovered in the nuclear fraction.

by filtration, equal volumes of both types of homogenates contained unequal amounts of DNA. However, when equal volumes of these were subjected to the nuclear isolation procedures through 2.3 M sucrose, both the absolute and percentage yields of nuclei (based on DNA measurements) were increased markedly when filtered homogenates were used. Nuclear recoveries are severely affected by particulate material isopycnic at the 1.62/2.3 M sucrose interface because this material acts as a physical barrier to the sedimentation of nuclei. Moreover, adequate filtration removes a substantial amount of this material that would sediment more rapidly than nuclei to this interface. Therefore, it is not surprising that both absolute and relative yields are improved when filtered homogenates are used.

The method of filtration adopted for use in experiment III was based on these studies. However, nucleic acid concentrations were measured in both crude and filtered homogenates obtained from selected groups of animals of experiment III to determine whether filtration had differential effects for different dietary groups. These are shown for DNA (Figure 1) and RNA (Figure 2).

Figure 1

DNA concentrations in crude and filtered homogenates prepared from animals that had been fed 5% or 20% casein-containing diets for 0, 8, or 16 days. Aliquots of liver homogenates were analyzed for DNA prior to and after filtration as described in MATERIALS AND METHODS for experiment III. Squares, crude homogenates; circles, filtrates. 5% casein, □—□—□ or o—o—o; 20% casein, ■—■—■ or ●—●—●; zero time controls, ⊠ or ⊗.

Insert. Percentage decrease in apparent DNA concentration attributable to the filtration procedure for various treatment groups. 5% casein, open bars; 20% casein, closed bars; zero time controls, hatched bar.

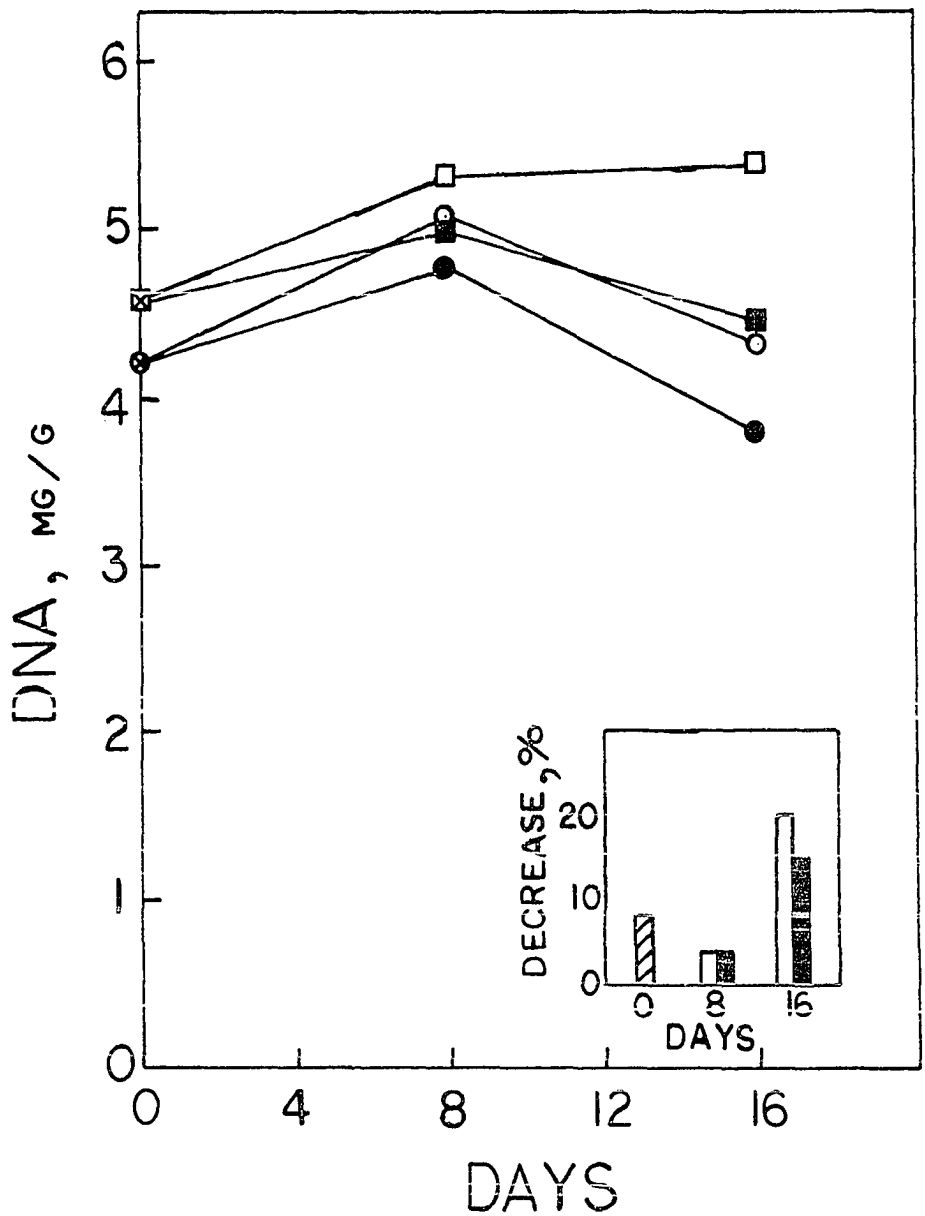
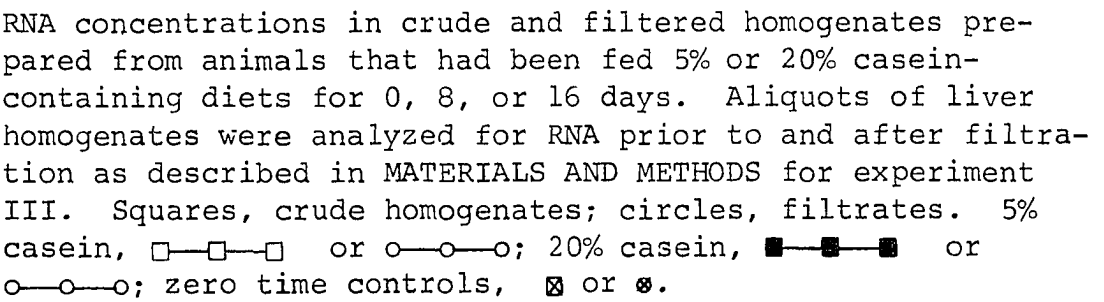
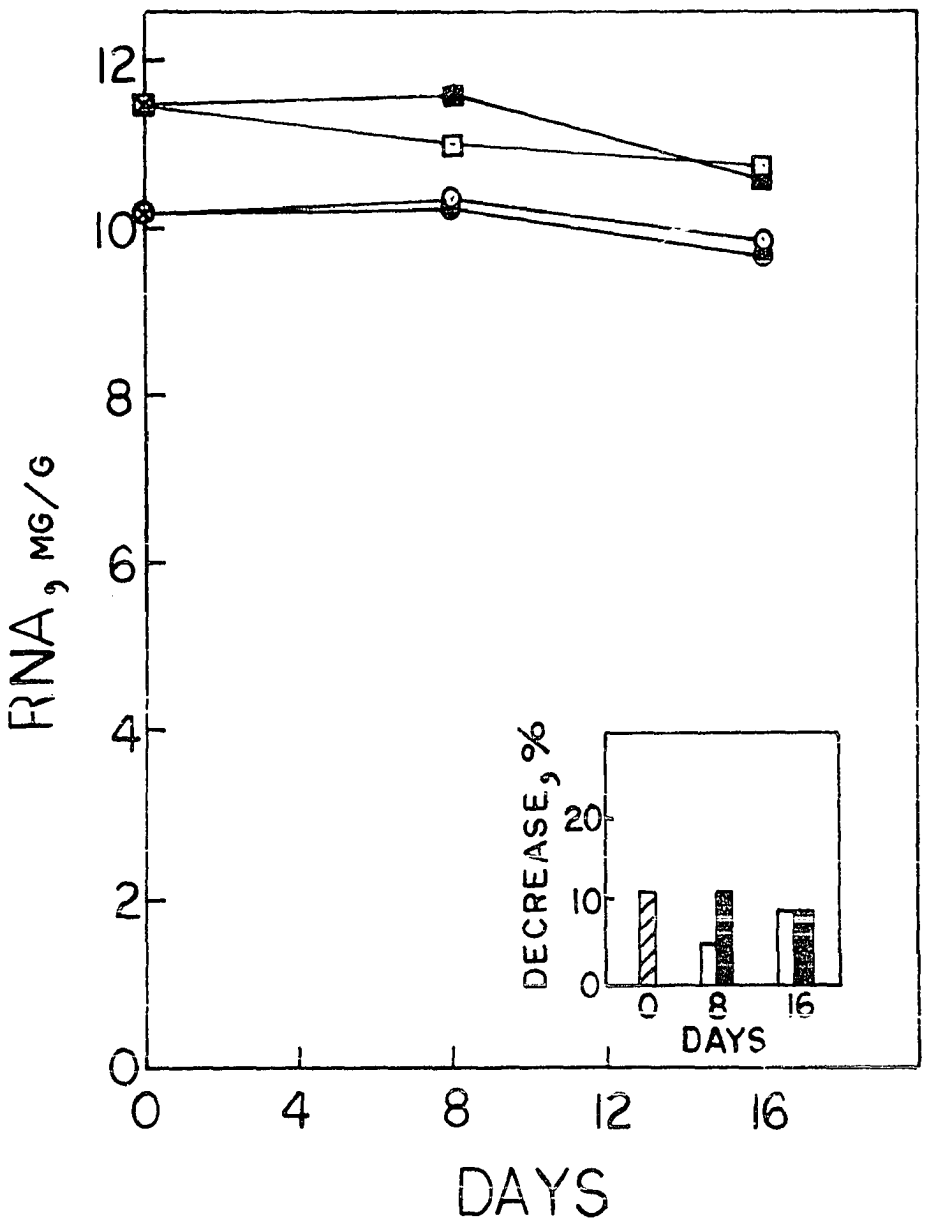

DNA, MG/G
0
1
2
3
4
5
6
0
4
8
12
16
DAYS
DECREASE, %
0
10
20
0
8
16
DAYS

Figure 2



RNA concentrations in crude and filtered homogenates prepared from animals that had been fed 5% or 20% casein-containing diets for 0, 8, or 16 days. Aliquots of liver homogenates were analyzed for RNA prior to and after filtration as described in MATERIALS AND METHODS for experiment III. Squares, crude homogenates; circles, filtrates. 5% casein, □—□—□ or o—o—o; 20% casein, ■—■—■ or o—o—o; zero time controls, ⊠ or ⊗.

Insert. Percentage decrease in apparent RNA concentration attributable to the filtration procedure for various treatment groups. 5% casein, open bars; 20% casein, closed bars; zero time controls, hatched bar.


RNA, MG/G
0
2
4
6
8
10
12
0
4
8
12
16
DAYS
DECREASE, %
0
10
20
0
8
16
DAYS

In Figure 1, two homogenates of each treatment group fed 5% or 20% casein for 0, 8, or 16 days were analyzed for DNA using crude (unfiltered) and filtered homogenates. No differences in the concentration of DNA between dietary groups was apparent at any time period. Similarly measurements for RNA are illustrated in Figure 2; on an average approximately 10% of the cellular RNA was removed by filtration, and differences between dietary groups appear to be within the limits of reproducibility of the methods.

D. Chemical Determinations

Tissue nucleic acids were precipitated by 0.2 N $HClO_4$, and RNA and DNA were separated by alkaline hydrolysis according to the Schmidt-Thannhauser procedure as recommended by Munro and Fleck (1969). Hydrolyzed RNA was estimated by absorbancy at 260 nm (Munro and Fleck 1966), and DNA was estimated according to Ceriotti (1955) as modified by Short, Warner, and Koerner (1968). Protein was determined according to Lowry et al. (1951).

1. RNA and DNA

Homogenates (filtered and unfiltered) were diluted prior to analysis by the addition of 15.7 ml TKM buffer per ml.

Aliquots of the nuclear fractions also were adjusted appropriately using TKM as diluent. Aliquots (5.0 ml equivalent to 100 mg wet wt liver) were acidified with $HClO_4$ to a final concentration of 0.2 N by the addition of 2.5 ml 0.6 N $HClO_4$. After 10 to 15 minutes at 0 C, precipitates of nucleic acid and protein were centrifuged at 1000 x g for 10 minutes and washed twice with 5 ml 0.2 N $HClO_4$. The precipitates were stirred into smooth pastes prior to dissolving them in 4 ml 0.3 N NaOH. Incubation at 37 C for one hour produced alkaline digests. After cooling to 0-4 C, DNA and protein were reprecipitated by the addition of 2 ml 1.4 N $HClO_4$. The supernatant after centrifugation contained ribonucleotides. Two successive washes of the precipitate with 5 ml 0.2 N $HClO_4$ were added to the supernatant and the total diluted to 50 ml with water. Absorbancy at 260 nm of the resulting solution of ribonucleotides in 0.1 N $HClO_4$ was measured. Using the extinction coefficient $E^{1\%}_{1cm}$ of 312 ($O.D._{260}$ = 1.000 corresponding to 32 ug RNA/ml), RNA was estimated (Munro and Fleck, 1966).

The residues remaining after precipitation and washing of the alkaline digest were dissolved in 25 ml 0.1 N NaOH and stored overnight at 5 C. DNA was determined colorimetrically

by the indole reaction as described originally by Ceriotti (1955) and modified by Short, Warner, and Koerner (1968). The latter modification utilizes 25 uM $CuSO_4$ in the final reaction mixture and permits linear standard curves over a wider range. Aliquots (2.0 ml) of test or standard DNA in 0.1 N NaOH were mixed with 1.0 ml 0.04% indole-100 uM $CuSO_4$ and 1.0 ml concentrated HCl in test tubes which were then covered with marbles to minimize evaporation while they were heated at 100 C for 10 minutes. These tubes were cooled rapidly in ice water and their contents were extracted twice with 6 ml chloroform. Absorbancies at 490 nm were measured, and the DNA content of samples was estimated using calf thymus DNA (Calbiochem, Los Angeles, California) as a reference.

2. Protein

Protein determinations were performed on aliquots of the 0.1 N NaOH extracts, described above, by the method of Lowry et al. (1951). Aliquots of test or standard protein (1.0 ml) in 0.1 N NaOH was mixed with 5 ml of reagent C which was freshly prepared from one volume 0.5% $CuSO_4$, one volume 1% sodium potassium tartrate, and fifty volumes of 2% Na_2CO_3 in 0.1 N NaOH. After 10 minutes, 0.5 ml reagent E (Folin-Ciocalteau reagent diluted to 1 N acid) was added,

and the absorbancy at 550 nm was measured after 30 minutes. Bovine serum albumin (Pentex, Kankanee, Illinois) was used as reference.

E. Ribonuclease Assays

Ribonuclease activities of nuclei and homogenates were assayed in a system similar to that described by Bresnick, Sage, and Lanclos (1966). High molecular weight yeast RNA (Calbiochem, Los Angeles) was purified by gel filtration chromatography on sephadex G-100 in 0.01 M Tris-HCl pH 7.5. This purification was necessary to remove low molecular weight acid soluble ribonucleotides and thus reduce the substrate blank. Impurities in commercial RNA preparations that interfere with the enzyme assay were also removed (Roth and Wojnar 1961). For assay conditions, see legend of Figure 18.

F. Nuclear Counting Techniques

1. Hemocytometer method

Nuclear preparations of experiment II in 0.25 M sucrose-TKM were diluted 1:10 in 0.25 M sucrose containing 0.1% Azure C and were counted in a hemocytometer chamber (0.1 mm deep, Neubauer ruled) at 430 fold magnification. At least four replicates were counted for each sample. The total

number of nuclei in the central 0.1 mm^3 was multiplied by 10^5 to obtain the number of nuclei/cm^3 in the original preparation.

2. Electronic particle counting

Nuclear samples of experiment III were fractionated into diploid and tetraploid nuclei by sucrose gradient sedimentation (see below) in order to determine the average DNA/nucleus and to permit size distribution studies on each class. The grossly visible bands that corresponded to diploid and tetraploid nuclei were accurately located among the gradient fractions by measuring the absorbance at 260 nm of those fractions. Since the relative 260 nm absorbancies had been demonstrated previously to parallel the relative DNA concentrations among gradient fractions, (Separate experiment, results not shown) absorbance measurements provided both a precise and rapid identification of those fractions enriched in diploid and in tetraploid nuclei. Three tubes corresponding to the center of each grossly visible band of each nuclear class were then pooled for particle counting and size distribution analysis. Appropriate dilutions of the nuclei were made using Isoton (Coulter Electronics, Hialeah, Florida) as diluent prior to counting and particle

size distribution analyses using a Coulter Model B electronic particle counter. Instrument settings of AMP 1/4, APC 1/8, and a 100 u aperture were used and were shown previously to be adequate (Santen 1965) for studies on hepatocyte nuclei.

G. Statistical Analyses

Data were evaluated by analysis of variance according to Steel and Torrie (1960). For the studies described as experiment II, single degree of freedom orthogonal comparisons and minimum differences required for statistical significance ($P < 0.01$) were computed.

H. Sucrose Gradient Sedimentation Analyses

Nuclei were separated into diploid and tetraploid classes using the principle of velocity of sedimentation at unit gravity (Falzone, Barrows, and Yiengst 1962) and employing low speed centrifugation to permit more rapid separations (Albrecht 1968). Aliquots (2.0 ml) of nuclear suspensions were layered onto 49.5 ml linear sucrose gradients (15 to 40% w/v in TKM) in cellulose nitrate tubes (prepared at room temperature and equilibrated overnight at 4 C). After centrifugation at 4 C for 10 minutes at 1200 rpm (250 x g

average in the 240 rotor of a Size 1 International centrifuge), fractions (approximately 1.6 ml each) were collected from the bottom following puncture of the tube with an 18 gauge needle. After fractions were collected from the tube, any particulate matter at the bottom of the gradient tubes (presumably clumped nuclei or cell debris) was suspended in 1.6 ml 15% sucrose in TKM and represented fraction number 0. The DNA content of every fraction was determined by the method of Burton (1956) described below.

After the addition to each fraction of 1 ml carrier bovine serum albumin (1 mg/ml), the contents were made 0.5 N with respect to $HClO_4$ by the addition of 1.3 ml of 1.5 N $HClO_4$. After 30 minutes at 0 C, the precipitates were collected by centrifugation (10 minutes at 1000 x g), washed once with 5 ml 0.5 N $HClO_4$ to remove sucrose, and inverted to drain. The precipitates were suspended in 2.0 ml 0.5 N $HClO_4$, heated at 70 C for 15 minutes, cooled, and mixed with 4 ml diphenylamine reagent. After 14 to 18 hours at 30 C, traces of insoluble material were removed by centrifugation at room temperature (10 minutes at 1000 x g). Absorbancy at 600 nm was measured with calf thymus DNA used as reference standard.

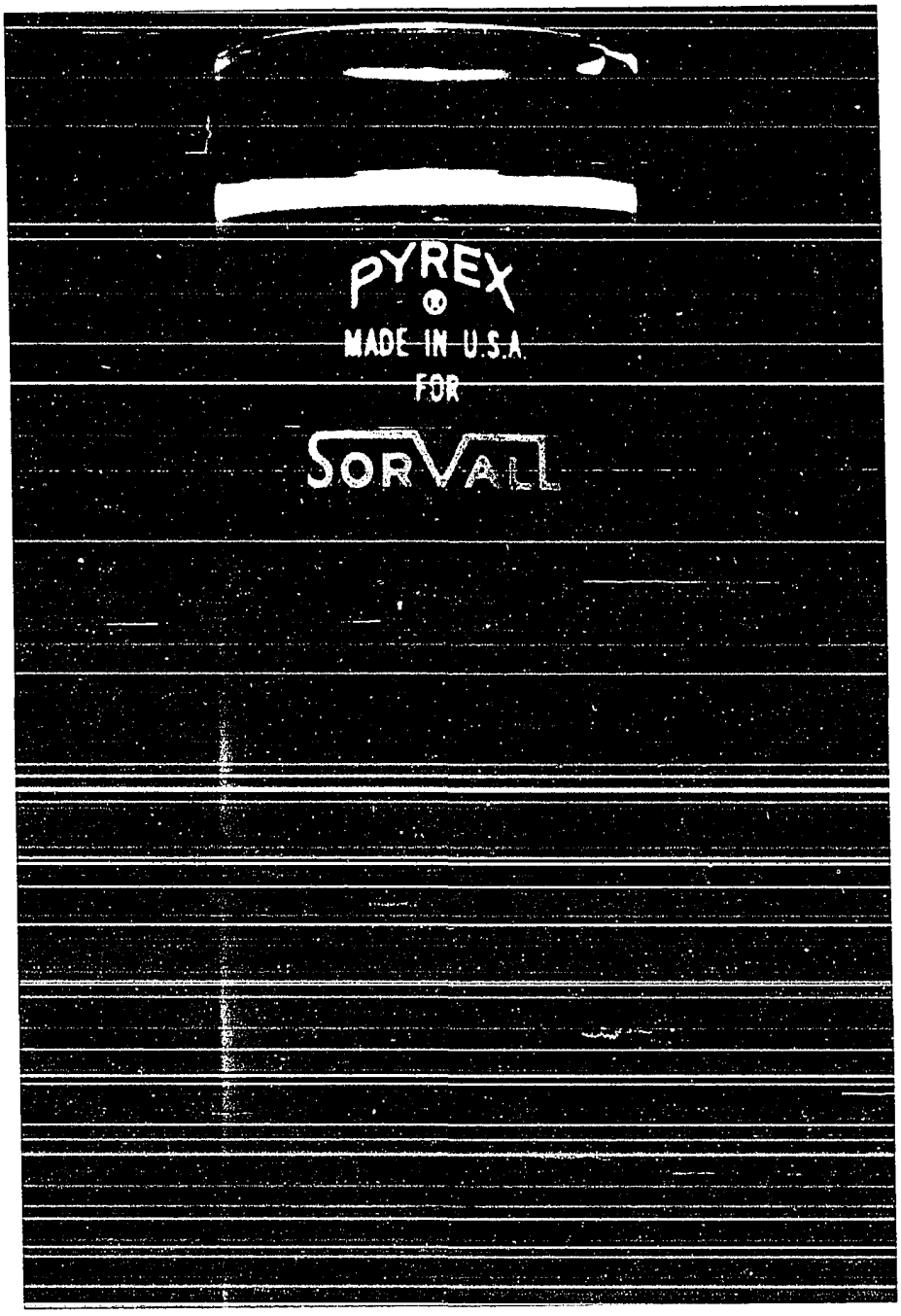
The major focus in experiment III was directed toward a different analytical approach to evaluate hepatic nuclear ploidy and the effect of protein restriction. Although the results of experiment II (cf Figure 10) did not demonstrate any significant dietary effects on nuclear ploidy, the validity of those data was questionable due to large variance and high absolute values for mean DNA/nucleus. Therefore, experiments were conducted to standardize methodology which would enable quantitative evaluation of hepatic nuclear ploidy by density gradient techniques.

When studies in this laboratory were performed using 15 to 40% w/v linear sucrose gradients combined with low speed centrifugation, separations could be effected in 10 minutes, and discrete bands corresponding to two major sedimentation classes of hepatic nuclei were grossly visible. The results of a typical separation, performed in a glass tube for photographic purposes, are shown in Figure 3 and illustrate the relative appearance of the major nuclear classes in the centrifuge tube at the end of the run.

When hepatic nuclei of 100 gram growing rats were subjected to this procedure in cellulose nitrate tubes and the tube contents were fractionated by tube puncture, the DNA

Figure 3

Separation of diploid and tetraploid nuclei by sucrose gradient centrifugation. Hepatic nuclei were isolated as described in MATERIALS AND METHODS for experiment III from a single liver of an animal that had received 20% casein for 16 days. The nuclei were suspended in 0.25 M sucrose - TKM buffer and layered above a 49.5 ml linear sucrose gradient (15-40% w/v) prepared in a glass centrifuge tube. Following centrifugation at 1200 rpm (250 x g, avg.) for 10 minutes, the tube was removed and photographed. Grossly visible bands midway in the tube correspond to diploid (upper) and tetraploid (lower) hepatic nuclei.


PYREX
MADE IN U.S.A.
FOR
SORVALL

was distributed into two major symmetrical peaks (Figure 4). These peaks corresponded to the grossly visible bands of turbidity shown previously in the photograph and consisted of a faster sedimenting class (peak at fraction #13) and a more slowly sedimenting class (peak at fraction #19). The DNA plotted at fraction zero represented a pellet recovered at the tube bottom presumably containing aggregates of nuclei. The DNA remaining near the miniscus (fractions 32 to 34) represented damaged or broken nuclei.

Since a prerequisite of reproducible gradient sedimentation is a reproducibly linear gradient, the linearity of a typical gradient under these conditions was investigated. Using methylene blue as a marker, gradients were prepared, centrifuged, and fractionated as described. As shown in Figure 5 the sucrose concentration in successive fractions was found to be acceptably linear over the entire gradient with minor deviations at the bottom and miniscus presumably due to mixing and diffusion.

Next, verification that the two sedimentation classes of nuclei were in fact diploid and tetraploid was attempted. Nuclei were separated into the two sedimentation classes, and for each class three independent criteria of ploidy were

Figure 4

DNA distribution in the sedimentation classes of hepatic nuclei obtained by sucrose density gradient centrifugation. Animals which had been fed an adequate diet of 20% casein for 16 days after weaning and which were growing at near optimal rates were utilized. Nuclei were isolated as described in MATERIALS AND METHODS for experiment III from the livers of four animals weighing between 107-120 g (average = 112) and having liver weights ranging between 3.27 - 4.10 g (average = 3.64). 2.0 ml of the nuclear suspension containing 1.30 mg DNA was layered on a 49.5 ml sucrose gradient (15-40% w/v) and centrifuged 10 minutes at 1200 rpm. The tube contents were fractionated by tube puncture and analyzed for DNA. Results are plotted as percent of DNA recovered from the gradient (recovery = 82%) and also as ug of DNA per gradient fraction. Direction of sedimentation is from right to left and fraction zero corresponds to material recovered as a pellet from the centrifuge tube bottom. Zone I, pellet at bottom of tube; Zone II, nuclei larger than tetraploid; Zone III, tetraploid nuclei; Zone IV, diploid nuclei; Zone V, light or damaged nuclei.

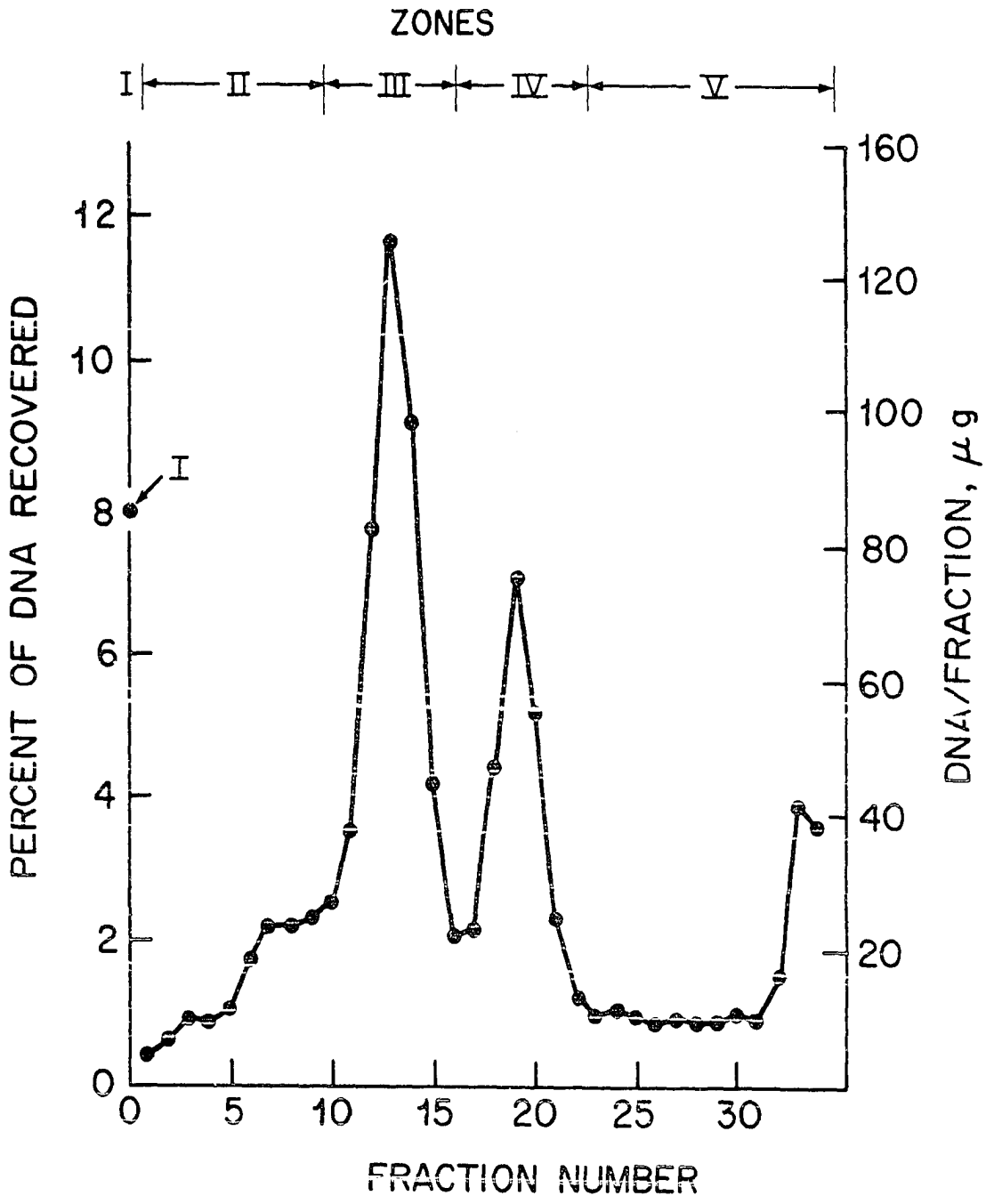
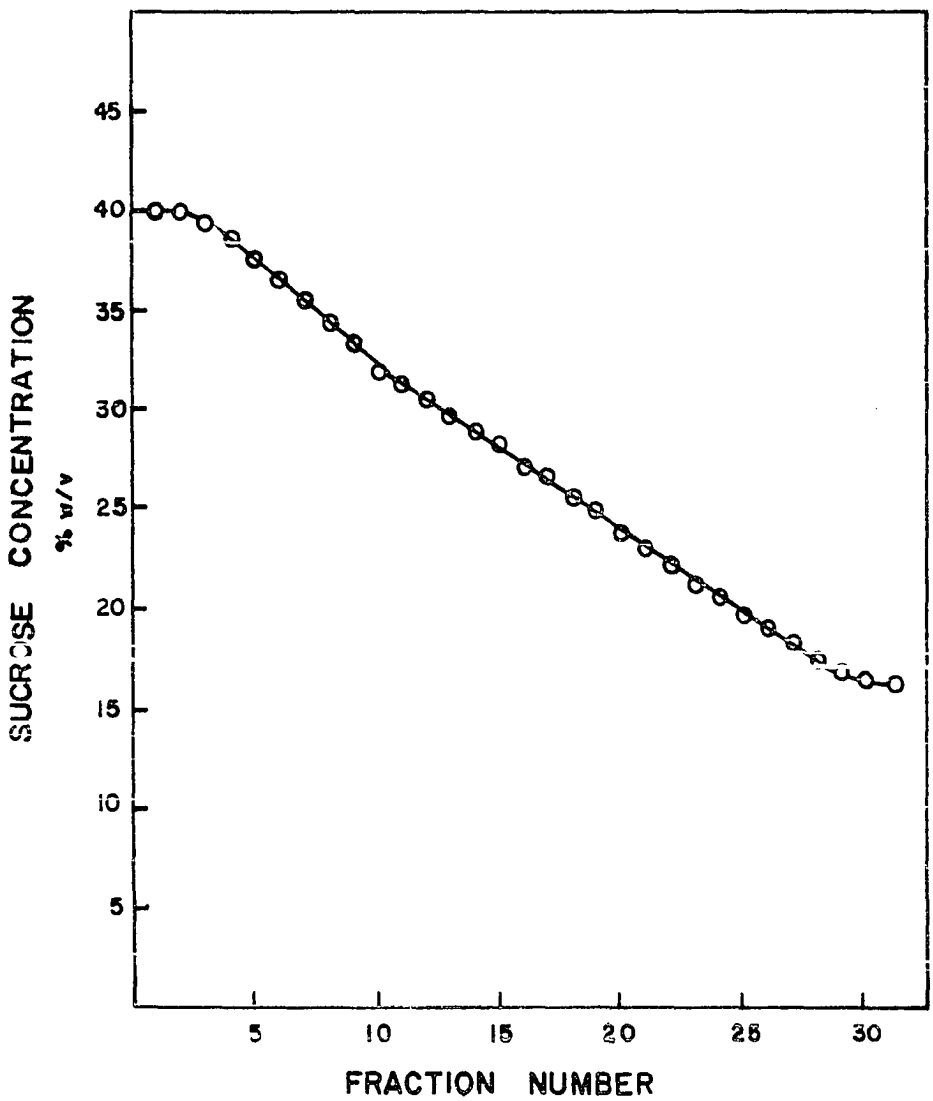

ZONES
I
II
III
IV
V
PERCENT OF DNA RECOVERED
DNA/FRACTION, μg
FRACTION NUMBER
0
2
4
6
8
10
12
20
40
60
80
100
120
160
0
5
10
15
20
25
30

Figure 5

Linearity of the sucrose concentration in a typical density gradient utilized in experiment III. A 49.5 ml sucrose gradient was prepared using a two-chambered mixing device from 26 ml of 15% w/v sucrose - TKM buffer and 23.5 ml of 40% w/v sucrose - TKM buffer containing methylene blue (0.01%). The gradient was chilled overnight at 4 C, centrifuged as described in MATERIALS AND METHODS and fractionated into 31 fractions (1.6 ml each). The methylene blue concentration in each fraction was estimated by measuring the extinction at 600 nm and relating the absorbancy to the sucrose concentration. The bottom of the tube is at left.


SUCROSE CONCENTRATION
% w/v
45
40
35
30
25
20
15
10
5
5
10
15
20
25
30
FRACTION NUMBER

assessed. These were (i) mean nuclear volumes obtained by calibrated microscopic measurements, (ii) size distribution studies utilizing a Coulter electronic particle counter and size plotter, and (iii) enumeration of the nuclei with a Coulter electronic particle counter and chemical estimates of their DNA content. These results are summarized in Figures 6-8.

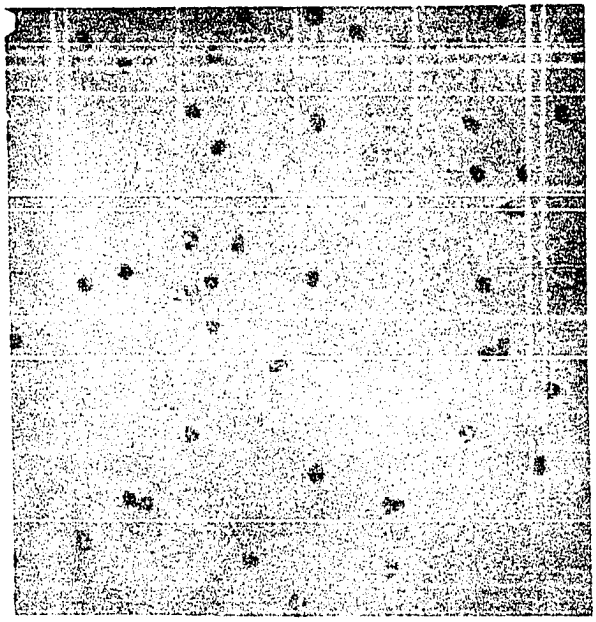
First, nuclei were separated into the two major sedimentation classes, and each class was examined microscopically with the use of a hemocytometer. Photomicrographs (Figure 6) were used for nuclear size measurements with a calibrated eyepiece and a dissecting microscope. Major and minor axes were estimated, and the volumes of nuclei were computed according to the formula for a prolate spheroid, $V = 4/3 \cdot ab^2$, where $2a =$ major axis and $b =$ minor axis (Falzone, Barrows, and Yiengst 1962). As indicated the mean nuclear volumes (u^3) were 517 ± 36 for tetraploid nuclei and 294 ± 20 for diploid nuclei. These results are in agreement with those of other workers who have separated nuclear classes by velocity sedimentation (Falzone, Barrows, and Yiengst 1962).

Secondly, size distribution studies of the two

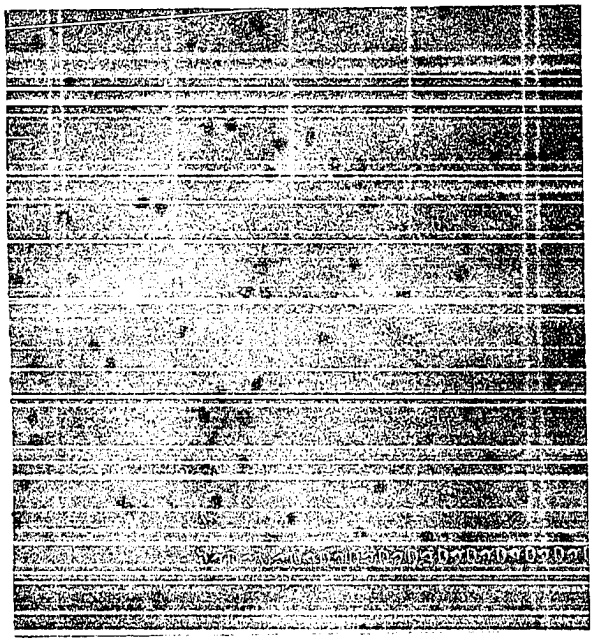
Figure 6

Photomicrographs of nuclei fractionated by sucrose density gradient centrifugation into tetraploid (A) and diploid (B) classes. Hepatic nuclei were fractionated as described in the legend of figure 4 . Aliquots of each grossly visible band were diluted in 0.25 M sucrose - TKM containing 0.1% Azure C and examined microscopically using a hemocytometer. Photomicrographs were made and measurements of the nuclei were obtained using a calibrated eyepiece and a dissecting microscope. The distance between grid lines is 50 u. The mean nuclear volume calculated for tetraploid nuclei (A) was 517 ± 36 and for diploid nuclei (B) was 294 ± 20 u^3.

A.



B.



sedimentation classes were undertaken. In Figure 7 are shown size distribution plots for diploid nuclei, tetraploid nuclei, and a mixture of both diploid and tetraploid nuclei. Recorder responses of the Coulter electronic particle counter are plotted as a function of increasing window number (corresponding to increasing volume). The difference in peak recorder responses is striking for the two classes and the distribution plot for a mixture of the two classes clearly exhibits a bimodal size plot. The validity of electronic particle counting techniques such as those described here has been evaluated (Santen 1965). When the peak recorder responses were superimposed in a single graph the clear cut separation of two discrete nuclear size classes was demonstrated (Figure 8).

Third, when samples of diploid and tetraploid nuclei were enumerated by Coulter electronic particle counting and analyzed chemically for DNA, values for pg DNA per nucleus were approximately 7 and 12, respectively. (Results not shown). This measure of ploidy is in agreement with other investigators and is consistent with the fact that the class identified as tetraploid nuclei contained twice the amount of DNA per nucleus as did that of the diploid class (Falzone,

Figure 7

Particle size distribution plots for the two classes of hepatic nuclei separated by sucrose density gradient centrifugation. Diploid nuclei, upper; tetraploid nuclei, middle; mixture of diploid and tetraploid nuclei, lower. Hepatic nuclei (from sample #15, experiment III) were fractionated by sucrose gradient centrifugation. The absorbance at 260 nm was used to locate the peak fraction corresponding to each class of nuclei, and aliquots (50 ul) of the peak fraction and one adjacent fraction on either side were pooled. Suitable dilutions were performed immediately prior to size distribution analysis using a Coulter electronic particle counter.

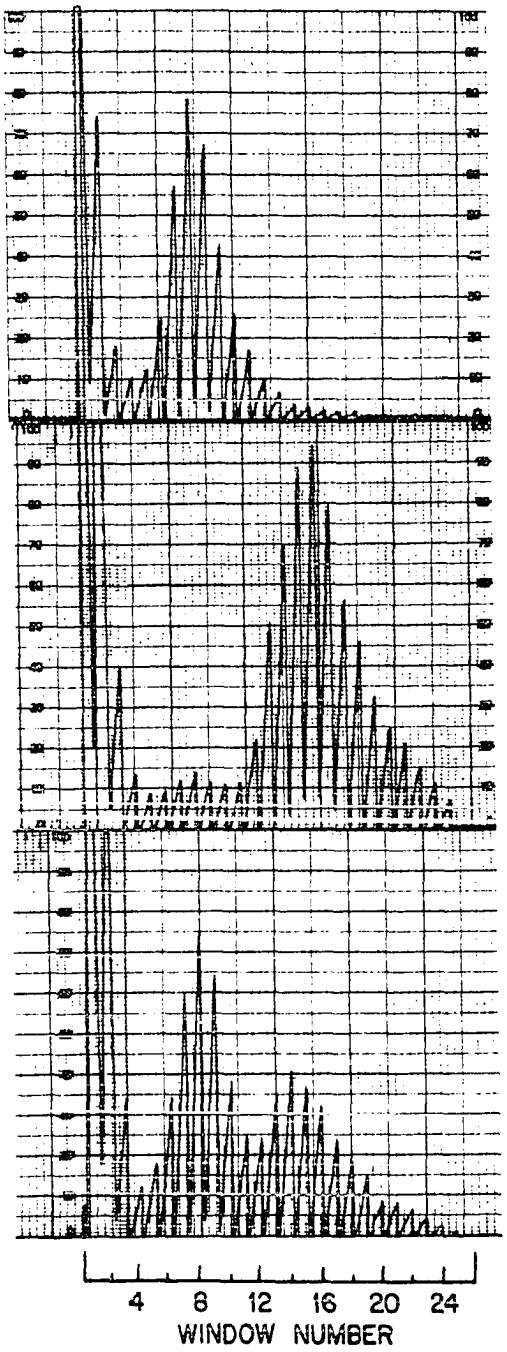
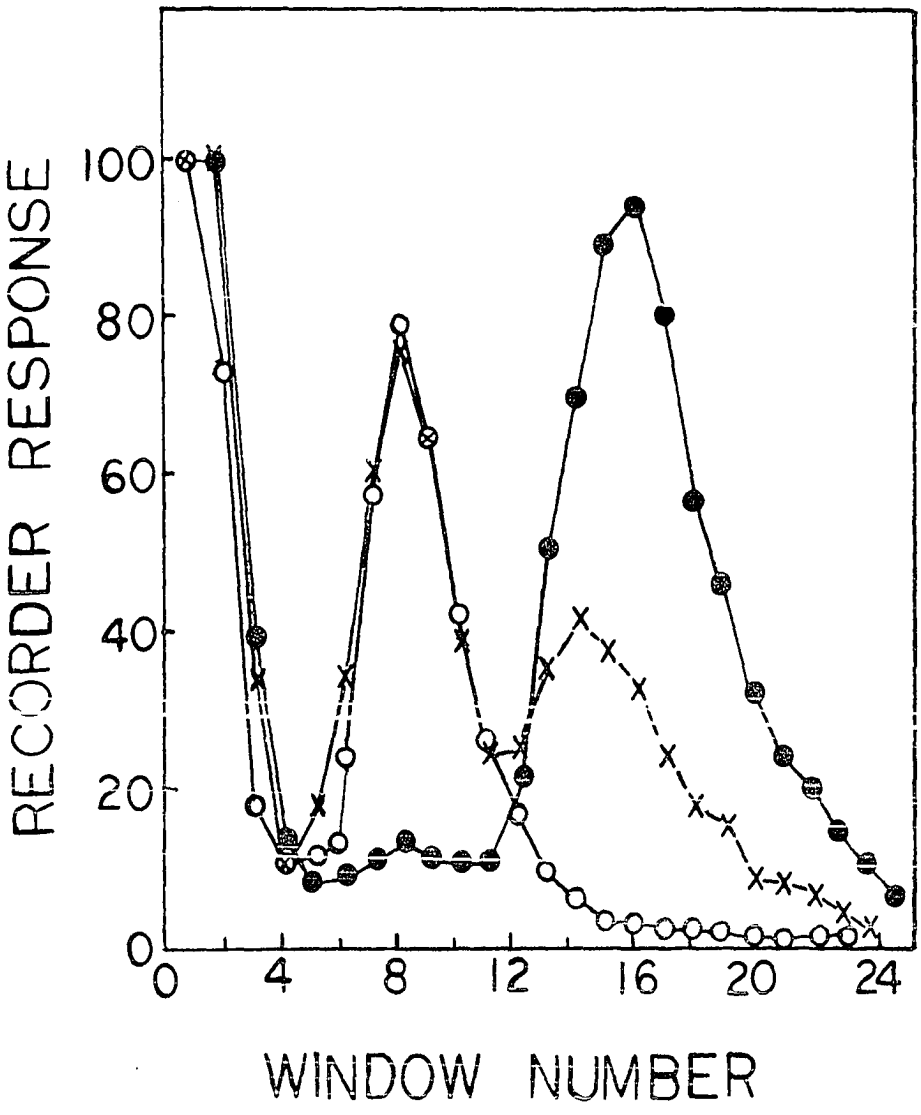

4
8
12
16
20
24
WINDOW NUMBER

Figure 8

Particle size distribution plots of diploid nuclei, tetraploid nuclei and a mixture of the two classes of nuclei. Data are taken from Figure 20 in which the peak recorder responses corresponding to each window setting have been plotted on a single graph. Diploid nuclei, o—o—o; tetraploid nuclei, ●—●—●; mixture of diploid and tetraploid nuclei, x—x—x.


RECORDER RESPONSE
100
80
60
40
20
0
0
4
8
12
16
20
24
WINDOW NUMBER

Barrows, and Yiengst 1962; Santen 1965). These preliminary studies were evidence that nuclei could be separated into discrete classes by density gradient centrifugation and that the major classes found in liver of rapidly growing weanling rats were diploid (2n) and tetraploid (4n) nuclei.

IV. RESULTS

A. Design of Experiments and Parameters Studied

Table VII summarizes the differences in the factorial design of three separate experiments conducted. To assess the time sequence of changes of various parameters following the feeding of diets containing 5 or 20% dietary protein, groups of animals were fed for 4, 8, 12, or 16 days. Values prior to feeding either diet were included in experiment III. Either 3 or 4 replicates were employed for each treatment group. Livers of 2 to 4 animals were pooled to provide sufficient tissue for analyses of total and nuclear hepatic constituents in experiments II and III. The total number of animals used varied from 32 to 144 in the three experiments.

The parameters studied in each experiment are summarized in Table VIII. Food intakes for the preceding 48 hours were measured every other day and body weights daily. Livers were weighed at autopsy. Protein, RNA, DNA, and ribonuclease were measured in hepatic homogenates and isolated nuclei. Hepatic nuclear ploidy was assessed by independent approaches in experiments II and III. Average ploidy was

Table VII. Differences in the factorial design[a] of experiments I, II, and III.

Statistic	Experiment		
	I	II	III
Number of treatment means	8	8	9[b]
Replicates within treatment means[c]	4	3	4
Number of animals within replicates[d]	1	2-4[e]	4
Number of animals per treatment mean	4	6-12	16
Total number of animals	32	81	144

[a] 2 x 4 factorial designs were used in all experiments and represent 2 levels of dietary protein (5% and 20%) and 4 time periods of feeding (4, 8, 12, and 16 days).

[b] Includes a zero time control.

[c] Number of homogenates analyzed for each treatment.

[d] Number of livers which were pooled for each homogenate.

[e] In experiment II, 4 livers (approximately 6 grams total wet weight of tissue) were pooled for each homogenate prepared from animals fed 5% casein (twelve animals per treatment mean). For animals fed 20% casein, the numbers of livers that were pooled to provide approximately 6 grams total wet weight of tissue were 4, 3, 2, and 2, respectively, for the time periods 4, 8, 12, and 16 days. The numbers of animals per treatment mean in the 20% casein groups were therefore 12, 9, 6, and 6 for these time periods.

Table VIII. Parameters studied in experiments I, II, and III.

	EXPERIMENT		
	I	II	III
Food Intakes, Growth, and Liver Weights			
Food intakes (measured on alternate days)	+	+	+
Body weights (measured daily)	+	+	+
Liver weights	+	+	+
Whole Cell Homogenates			
DNA	+	+	+
RNA	+	+	+
Protein	+	+	+
Ribonuclease		+	
Purified Nuclei			
DNA	+	+	+
RNA	+	+	
Protein	+	+	
Ribonuclease		+	
Nuclear Ploidy			
DNA / Nucleus (by chemical measurement and hemocytometer enumeration)		+	
Diploid and Tetraploid Distribution (by sucrose density gradient sedimentation analyses)			+

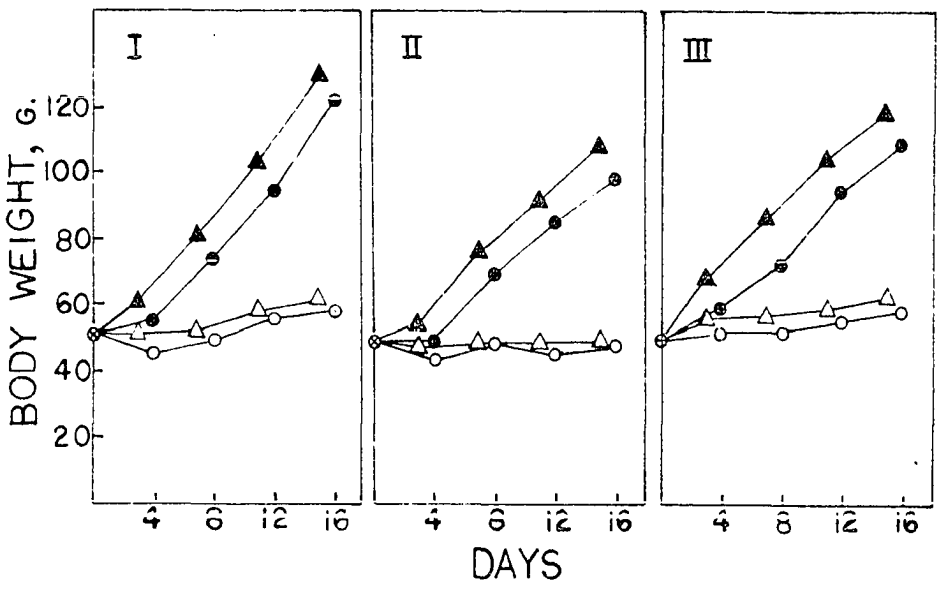
estimated in isolated liver nuclei by hemocytometer counting and chemical estimates of DNA in experiment II. In experiment III, liver nuclei were isolated and subjected to sucrose density gradient sedimentation analyses to separate diploid and tetraploid nuclei into discrete classes. The amount of DNA in each class was used to estimate the relative number of nuclei within each sedimentation class. In addition, the procedure for cheesecloth filtration of hepatic homogenates was standardized so that removal of fibrous and connective tissue elements was reproducible. As a consequence, nuclear recovery was increased.

B. Food Intake, Growth, and Liver Weights

Average body weights of the experimental animals are illustrated in Figure 9. Briefly stated, 20% casein supported optimal or near optimal growth whereas 5% casein feeding resulted only in the maintenance of body weight. Deviations from initial body weight in the low protein group were minimal, on an average, over the time course between zero and sixteen days. As can be seen in panels I, II, and III the apparent growth responses to each diet were similar in all three experiments.

Figure 9

Effect of the 18 hour fasting period prior to autopsy on the body weights of animals in experiments I, II, and III. Average body weights at autopsy (18 hours post-absorptive) are plotted for the time periods 4, 8, 12, and 16 days (circles). The average body weight of the same group 24 hours previously (absorptive) are plotted for the time periods 3, 7, 11, and 15 days (triangles). 5% casein, ○—○—○; or △—△—△; 20% casein, ●—●—● or ▲—▲—▲; ⊗, initial body weight.


I
II
III
BODY WEIGHT, G.
120
100
80
60
40
20
4
8
12
16
DAYS

Liver weights differed even more between the 5% and 20% casein groups than did body weights. Ratios of liver weight to body weight, illustrated in Figure 10, were consistently lower in those animals receiving 5% dietary protein than in those fed 20% protein. On an average, liver weights approximated 3.5% of the total body weight at all time periods for the control animals fed 20% casein, and tended to fall to values approaching 3.0% during the feeding periods for the animals fed 5% casein.

Comparative data for initial age and weight, growth rate, and food intake are tabulated in Table IX. Only the data for the final 12 days (4 to 16 days) of the experimental periods have been reported in detail here. The time period zero to four days was not included due to the initial period required for adaptation to the diet after weaning. Average growth rates were estimated by least squares regression analyses of the body weight data of Figure 9, using the mean body weight at autopsy (18 hours post-absorptive).

Animals receiving 5% casein gained body weights at average rates of 0.23 to 0.97 grams per day. The growth response to diets containing 20% casein varied in three experiments between 4.10 and 5.56 grams per day.

Figure 10

Liver weight/body weight ratios for experiments I, II, and III in response to feeding two amounts of dietary protein. 5% casein, o—o—o; 20% casein, ●—●—●; zero time controls of experiment III, ⊗.

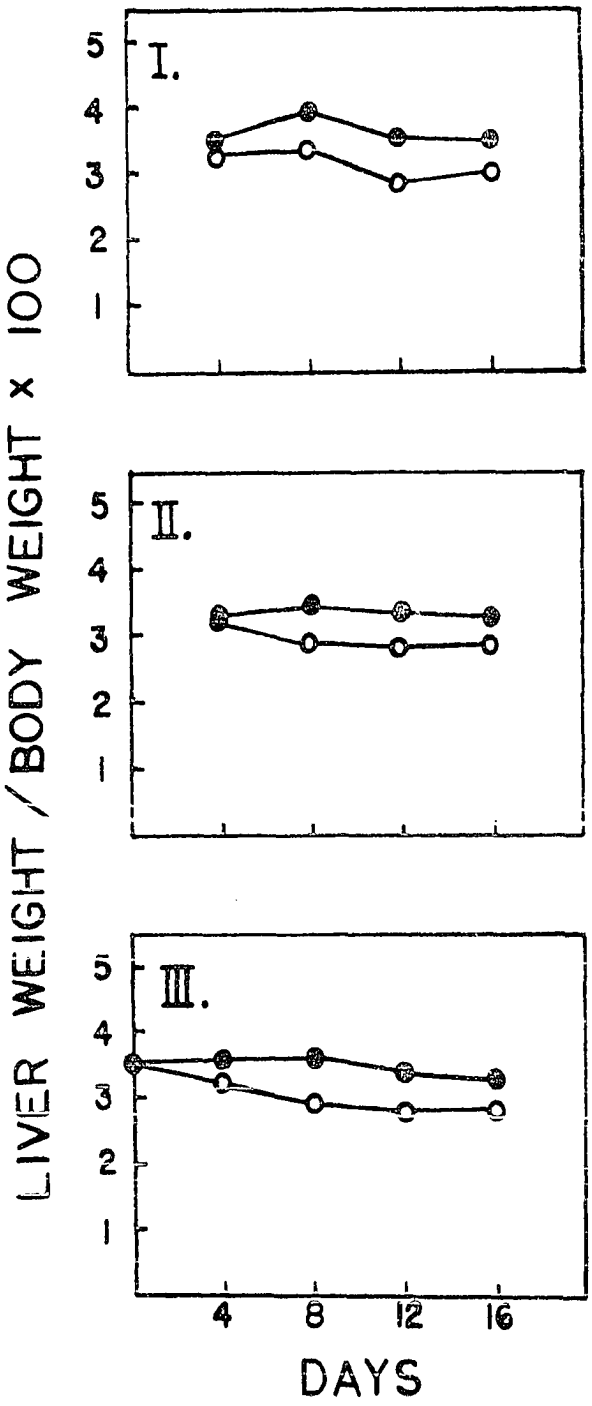

LIVER WEIGHT / BODY WEIGHT x 100
I.
II.
III.
5
4
3
2
1
4
8
12
16
DAYS

Table IX. Comparative data for initial age and weight, growth rate, and food consumption for the time period four to sixteen days in experiments I, II, and III.

Diet	Experiment	Initial[a] Age (days)	Initial[a] Body Weight (g)	Growth Rate[b]	Food Intake	
				$g \cdot day^{-1}$	$g \cdot day^{-1}$	$g \cdot day^{-1} \cdot 100g$ body $weight^{-1}$
5% Casein	I	23.8	51.1 ± 0.36[c]	0.97	6.16	10.8
	II	21.5	48.6 ± 0.16	0.23	3.87	7.8
	III	--[d]	54.8 ± 0.20	0.50	5.88	10.2
20 % Casein	I			5.56	11.05	13.0
	II			4.10	8.03	11.0
	III			4.25	8.48	10.6

[a] Average of all animals used.

[b] Average daily changes in body weights were computed for the time period 4 to 16 days by least squares regression analysis of the growth curves shown in Figure 9.

[c] Standard error of the mean.

[d] Age not provided by the supplier.

Absolute values for food intake (grams per day) for animals fed 20% casein were approximately twofold greater than for those fed 5% casein (11.05 vs. 6.16; 8.03 vs. 3.87; 8.48 vs. 5.88). However, for food intakes expressed per unit body weight, the striking differences between dietary groups are reduced.

C. Liver Composition

The data for DNA, RNA, and protein have been obtained from measurements of hepatic homogenates of 2 to 4 pooled livers, and three or four separate homogenates at each time period were analyzed. The results are summarized in Figures 11, 12, and 13. Both the concentration (mg/g) and content (mg/liver) have been plotted. The difference between treatment means accepted as statistically significant ($P<0.01$) is indicated. Mean concentrations are also in tabular form (Table X); for summaries of each analysis of variance of these data, see APPENDIX.

Concentrations of DNA (Figure 11) were higher at all time periods in livers of animals fed 5% casein than in those animals fed 20% casein. These differences were significant only on the 8th and 12th day in experiment II. In experiment III, although qualitatively similar changes were noted, both

Figure 11

Hepatic DNA concentration (mg/g) and content (mg/liver) in response to feeding the casein-containing diets. 5% casein, o—o—o; 20% casein, ●—●—●; zero time control ⊗; the minimum significant difference ($P < 0.01$) is indicated by the open bar.

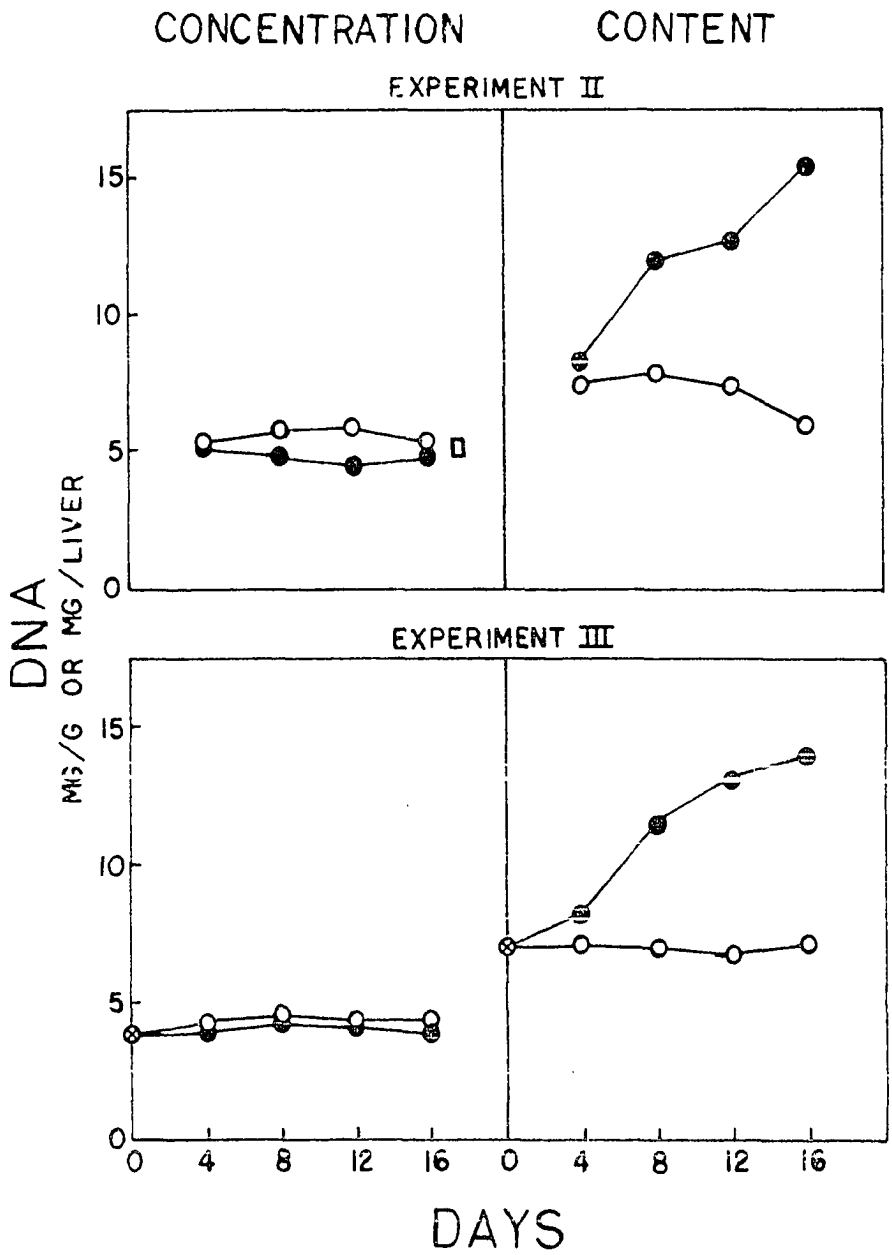

CONCENTRATION
CONTENT
EXPERIMENT II
EXPERIMENT III
DNA
MG/G OR MG/LIVER
15
10
5
0
0
4
8
12
16
DAYS

Figure 12

Hepatic RNA concentration (mg/g) and content (mg/liver) in response to feeding the casein-containing diets. 5% casein, o—o—o; 20% casein, ●—●—●; zero time control ⊗; the minimum significant difference ($P < 0.01$) is indicated by the open bar.

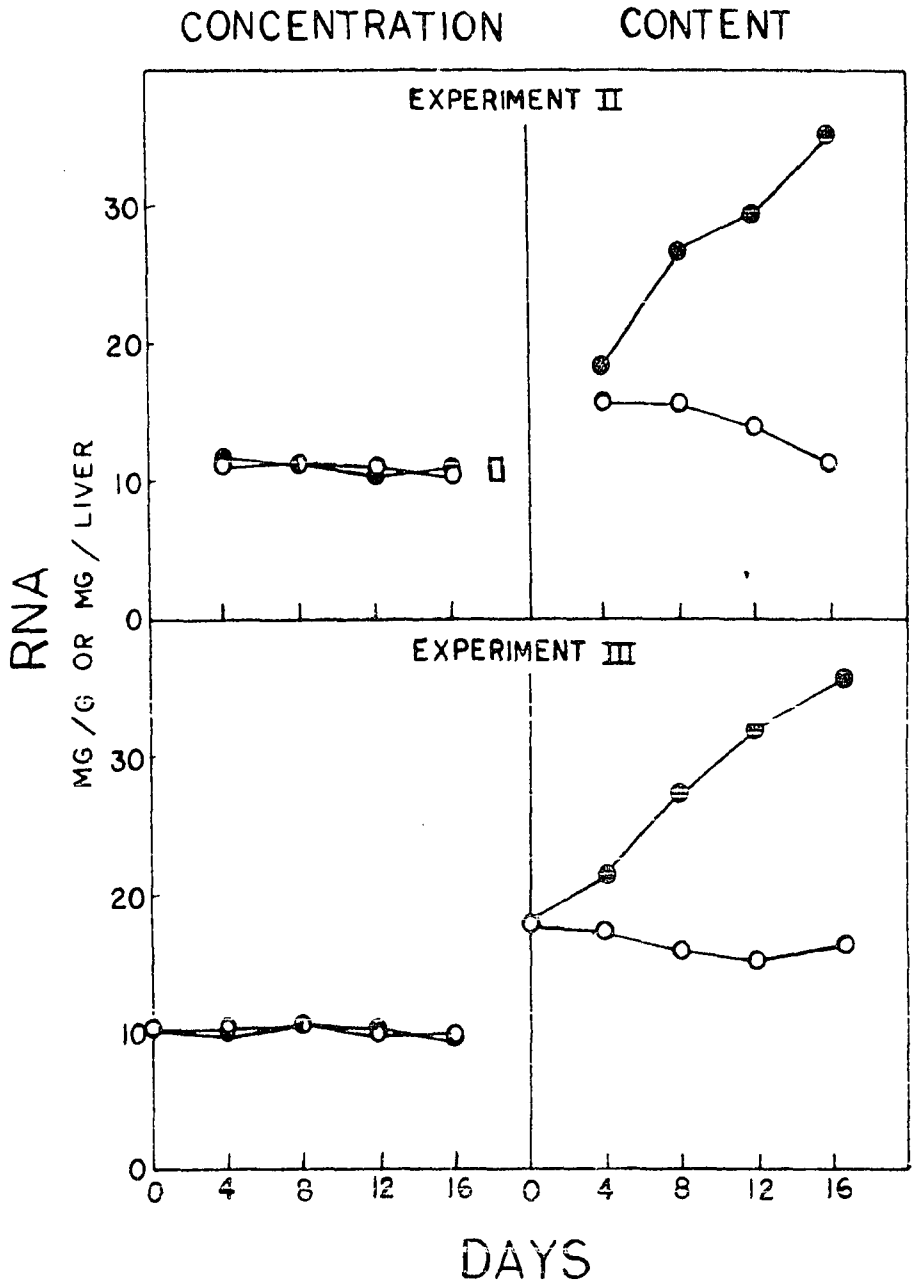

CONCENTRATION
CONTENT
EXPERIMENT II
EXPERIMENT III
RNA
MG / G OR MG / LIVER
0
10
20
30
0
4
8
12
16
DAYS

Figure 13

Hepatic protein concentration (mg/g) and content (mg/liver) in response to feeding the casein-containing diets. 5% casein, o—o—o; 20% casein, ●—●—●; zero time control ⊗; the minimum significant difference ($P < 0.01$) is indicated by the open bar.

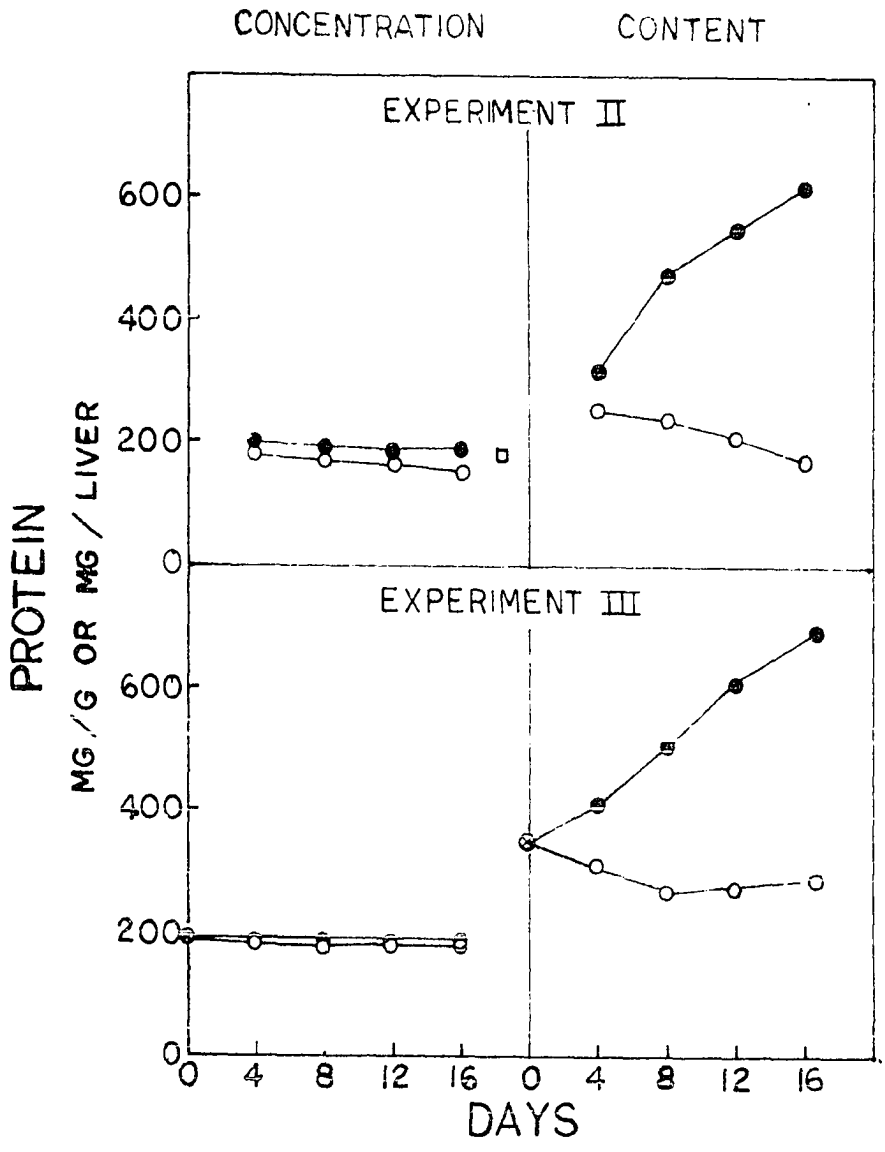

CONCENTRATION
CONTENT
EXPERIMENT II
EXPERIMENT III
PROTEIN
MG./G OR MG./LIVER
600
400
200
0
0
4
8
12
16
DAYS

Table X. Average concentrations (mg/g) of hepatic DNA, RNA, and protein[a,b]

Experiment	5% Casein DAYS 0	4	8	12	16	20% Casein DAYS 0	4	8	12	16	Minimum Significant Difference ($P < 0.01$)
					DNA (mg/g)						
II		5.31	5.72	5.73	5.36		5.12	4.90	4.45	4.85	0.56
III	3.94	4.26	4.58	4.41	4.41	3.94	3.84	4.35	4.09	3.88	0.85
					RNA (mg/g)						
II		11.1	11.1	10.8	10.1		11.3	11.1	10.3	11.0	1.18
III	10.1	10.3	10.5	9.8	10.0	10.1	10.0	10.3	9.9	9.9	0.72
					PROTEIN (mg/g)						
II		178	173	162	150		198	196	192	194	24.1
III	196	184	178	181	190	196	190	192	190	193	14.8

[a]For experiment II values represent the average composition measured in three homogenates. Since the homogenates were prepared from 2-4 pooled livers for each treatment the total number of animals used in each treatment varied from six to twelve (see Table VII).

[b]For experiment III, values represent the average composition measured in four homogenates and each homogenate represented four pooled livers.

the absolute concentrations of DNA and the magnitude of differences in concentrations was smaller than observed in experiment II. The lower apparent DNA concentrations in experiment III presumably may be attributable to the method of cheesecloth filtration of homogenates used in the studies of experiment III (For example, the effectiveness of filtration was evaluated previously in detail (see Materials and Methods, III.C.), and the apparent concentrations of hepatic constituents were computed on the assumption that one gram wet weight of liver was equivalent to 3.0 ml of 33% w/v homogenate, whether filtered or unfiltered). Total liver DNA paralleled liver weights.

RNA concentrations, shown in Figure12, did not differ between dietary groups at any time period studied nor did they change significantly with time in either dietary group suggesting that alterations in total cellular RNA closely parallelled hepatic weight.

Protein concentration measurements in hepatic homogenates from the two dietary treatment groups are shown in Figure 13. In experiment II the concentration of liver protein was lower at all time periods in animals fed 5% casein than in those fed 20% casein, but statistical significance occurred

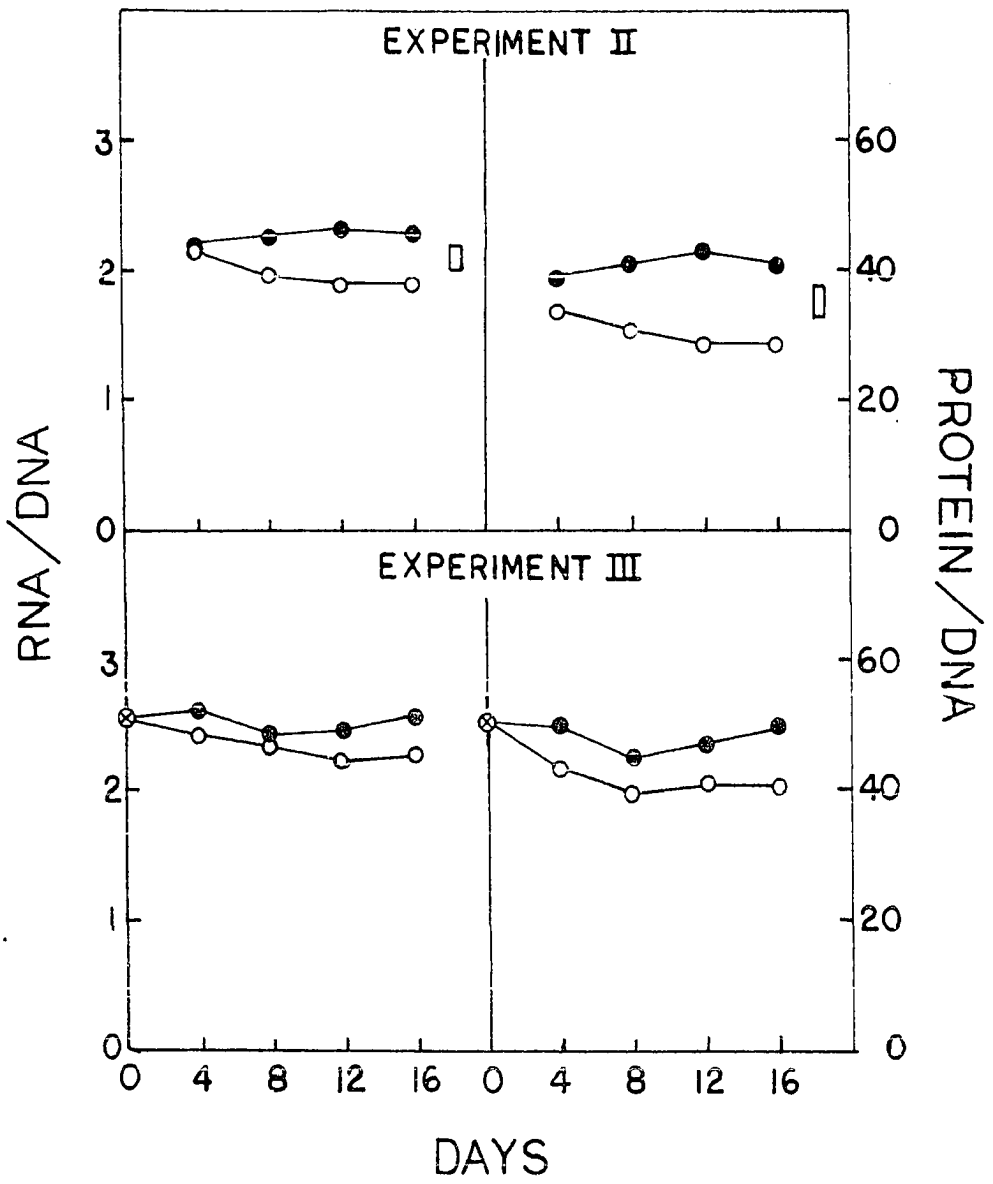
between dietary groups only on the 12th and 16th days. In experiment III neither differences in hepatic protein concentration due to diet nor to time occurred.

Because the changes in both RNA and protein concentrations due to the amount of casein in the diet occurred in an opposite direction to changes in DNA concentrations, alterations in the ratios of RNA and protein to DNA (Figure 14) are amplified. In experiment II, these ratios remained constant over the time periods studied for animals receiving 20% casein while time-dependent losses of RNA relative to DNA resulted from feeding 5% casein. Differences were significant at 8, 12, and 16 days. Reduction in protein relative to DNA was significant at each time period tested in experiment II. Similar changes in the RNA/DNA and protein/DNA ratios were found in experiment III, although they were of lesser magnitude.

In summary, these time sequence studies suggested that several qualitative and quantitative differences resulted from relatively short term feeding of 5% casein. First, concentrations of DNA increased, of RNA remained constant and of protein decreased. Second, ratios of RNA/DNA were moderately reduced whereas those of protein/DNA were

Figure 14

Ratios of RNA/DNA and protein/DNA in response to feeding the casein-containing diets. 5% casein, o—o—o; 20% casein, ●—●—●; zero time control ⊗; the minimum significant difference ($P < 0.01$) is indicated by the open bar.


EXPERIMENT II
EXPERIMENT III
RNA/DNA
PROTEIN/DNA
DAYS
0
4
8
12
16
20
40
60

markedly diminished. Third, adaptive changes occurred gradually but major alterations were complete by 8 or 12 days. The gross compositional changes found in the present study are less extreme and less acute than those reported by others for more extreme degrees of protein deficiency. However, they are compatible with previous observations that DNA concentrations are elevated in protein deficiency (Umana 1965; 1967), and concomitantly total cellular RNA (Munro, Naismith, and Wikramanayake 1953; Munro and Clark 1960) and protein (Allison and Wannemacher 1965; Lal, Agarwal, and Shankar 1975) are markedly decreased.

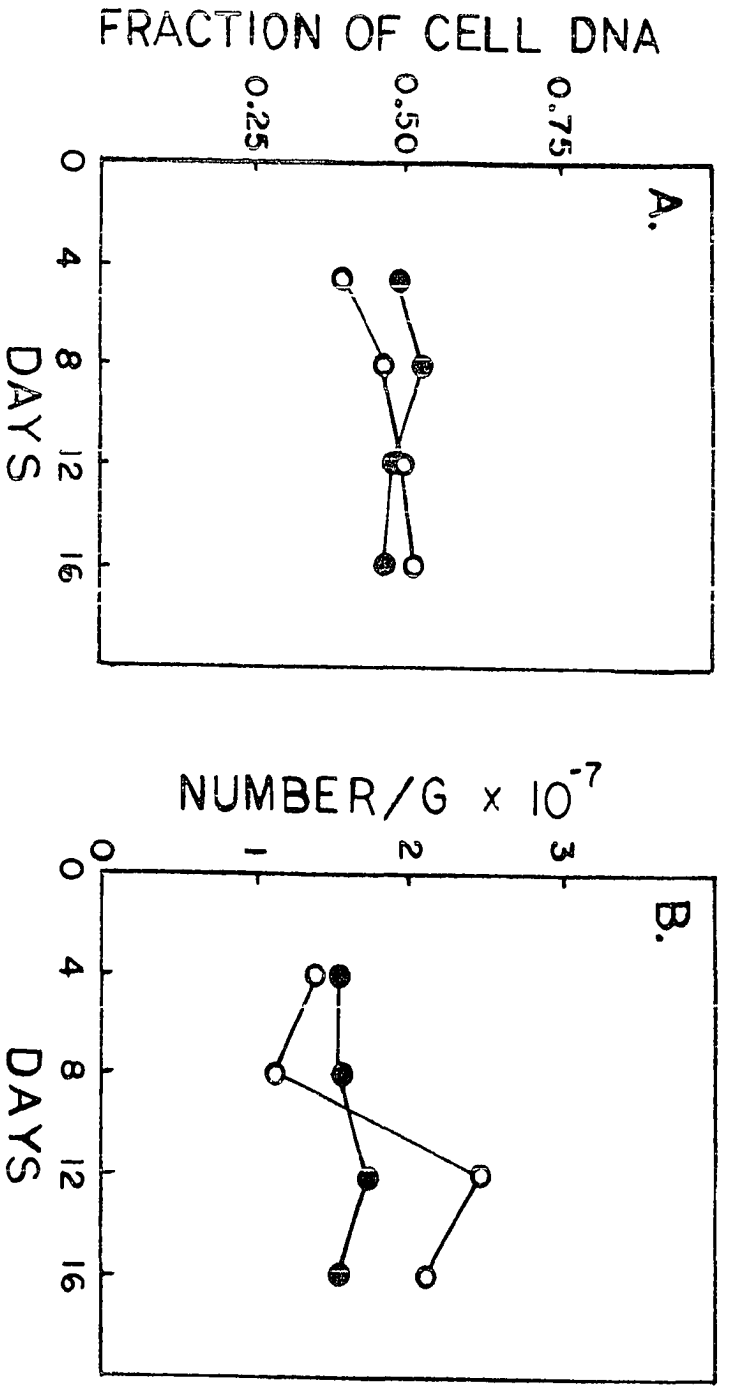
D. Composition of Liver Nuclei

Studies on nuclear composition similar to those for total hepatic constituents were also performed. Two empirical requirements of such studies are (i) that the isolation of nuclei must be representative of the total cellular population and (ii) that the nuclei thus isolated must be reasonably free from cytoplasmic contamination.

The results shown in Figure 15 suggested that reasonable recoveries of hepatic nuclei were obtained based on recovery of cell DNA (left panel, A) and by hemocytometer enumeration

Figure 15

Recoveries of hepatic nuclei estimated as a fraction of total cell DNA (A) and by enumeration utilizing hemocytometer counting (B). Hepatic nuclei were isolated as described in MATERIALS AND METHODS. DNA determinations performed on aliquots of homogenate and nuclei were used to calculate fractional recovery of total cell DNA. Nuclei were enumerated from aliquots of the nuclear fraction following staining with Azure C and the measurement expressed as number of nuclei isolated per gram of liver. 5% casein, o—o—o; 20% casein, ●—●—●.


FRACTION OF CELL DNA
0.25
0.50
0.75
A.
0
4
8
12
16
DAYS
NUMBER/G x 10^-7
0
1
2
3
B.
0
4
8
12
16
DAYS

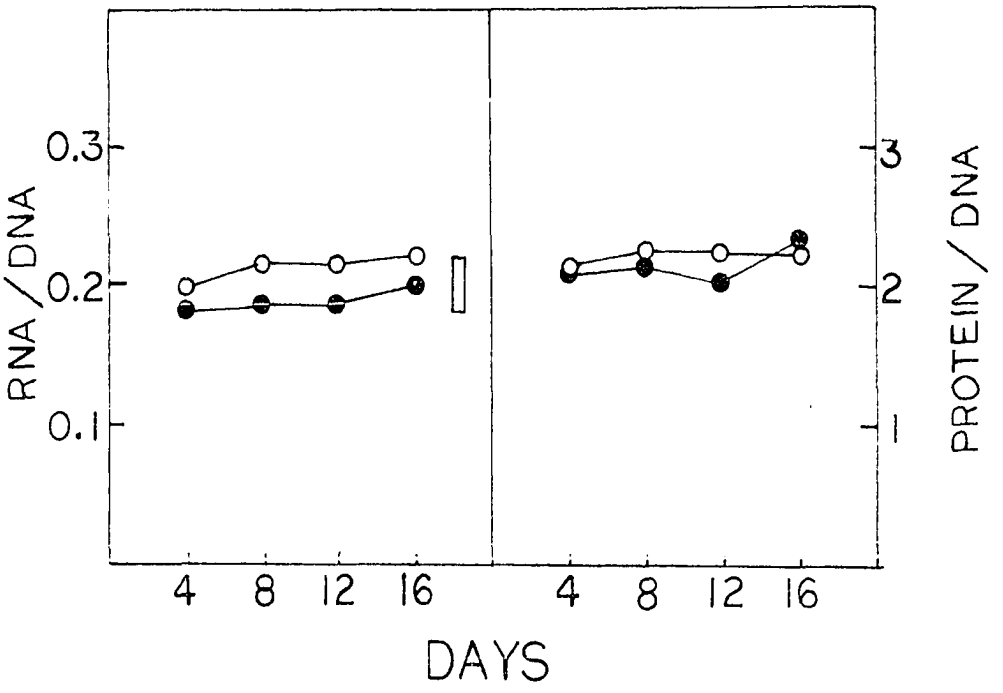
(right panel, B). From 40 to 50% of the cellular DNA was recovered, and differences in recovery between dietary treatment groups were minimal. Results based on hemocytometer estimates showed larger but nonsignificant differences between dietary groups. Nuclear fractions, therefore, were assumed to represent the hepatic parenchymal cell population in both dietary groups.

Although marker enzymes were not employed to evaluate the purity of hepatic nuclei, the RNA/DNA ratio was used as a criterion for estimating nuclear purity. The ratios in these studies approximated 0.2 for all treatment groups and were consistent with acceptable nuclear purity (Munro, Waddington, and Begg 1965; Blobel and Potter 1966; Tata 1974). The lack of effect of the dietary treatments imposed in these studies on hepatic nuclear composition is summarized in Figure 16. Neither RNA/DNA nor protein/DNA ratios were significantly altered by diet under the conditions of experiment II. Of possible interest and unknown significance is the time-dependent increase in RNA/DNA ratios for both dietary groups (significant by analysis of variance).

Measurements of DNA in isolated nuclei and enumeration of nuclei by hemocytometer counting were performed in an

Figure 16

Lack of effect of dietary protein on the composition of hepatic nuclei of animals fed 5% or 20% casein for various time periods (from experiment II). 5% casein, o—o—o; 20% casein, ●—●—●. The minimum significant difference ($P < 0.01$) is indicated by the open bar.


RNA / DNA
0.3
0.2
0.1
PROTEIN / DNA
3
2
1
4
8
12
16
4
8
12
16
DAYS

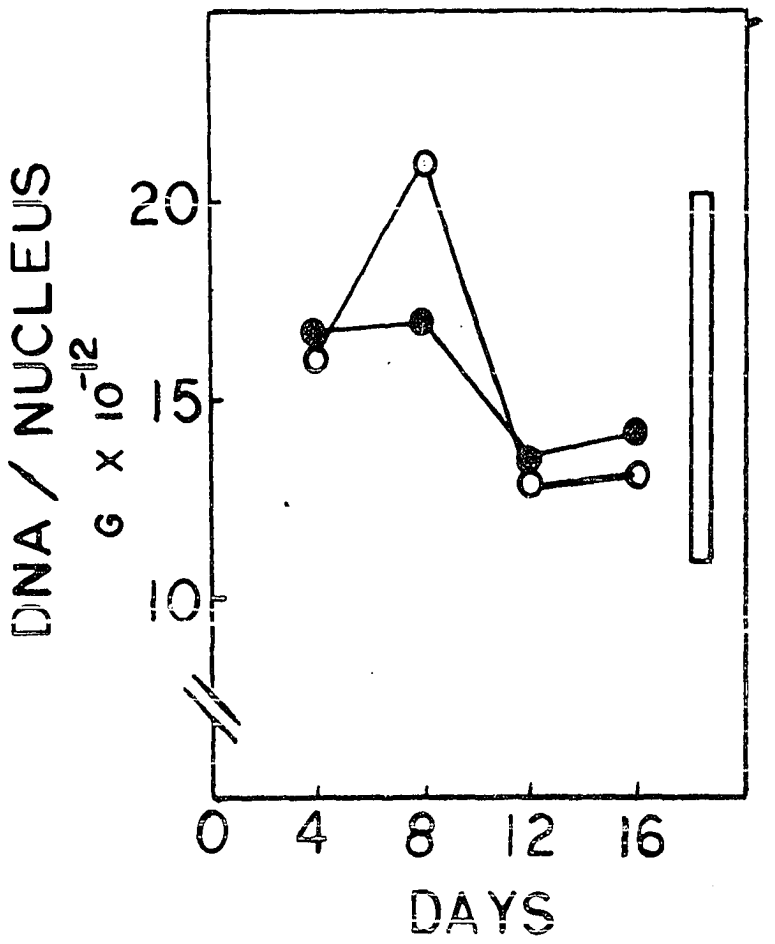
attempt to estimate dietary effects on average nuclear ploidy in experiment II. Shown in Figure 17 are the apparent average ploidies (pg DNA/nucleus) measured for the various treatment groups. Average ploidy did not differ statistically between animals fed 5% or 20% casein up to 16 days. The large variations encountered in estimating nuclei by hemocytometer techniques were largely due to clumping problems which were not controlled in experiment II. This variance is represented by the magnitude of difference necessary (open bar) for any two treatment means to be significant. Furthermore, the average values ranged between 13 and 21 pg DNA per average nucleus, considerably above accepted values for diploid mammalian nuclei. It appears likely that the methodological difficulties due to clumping resulted in a variable underestimate of nuclear number and a proportionate overestimate in nuclear ploidy.

E. Ribonuclease

Studies were performed to evaluate hepatic homogenate and nuclear alkaline ribonuclease in the absence and presence of p-chloro-mercuri-benzoate (PCMB), an antagonist of the action of a naturally occurring cytoplasmic ribonuclease inhibitor. Assays were performed under conditions in which

Figure 17

Average ploidy (DNA/nucleus) estimated by hemocytometer enumeration and DNA determination for hepatic nuclei isolated from animals fed the two levels of dietary protein. Data were taken from experiment II. 5% casein, o—o—o; 20% casein, ●—●—●. The minimum significant difference ($P < 0.01$) is indicated by the open bar.


DNA / NUCLEUS
G x 10⁻¹²
20
15
10
0
4
8
12
16
DAYS

rates were proportional to enzyme, and corrections were made for rates in the absence of enzyme to correct for substrate instability and in the absence of substrate to correct for endogenous rates. Stability of the enzyme to freezing was not tested.

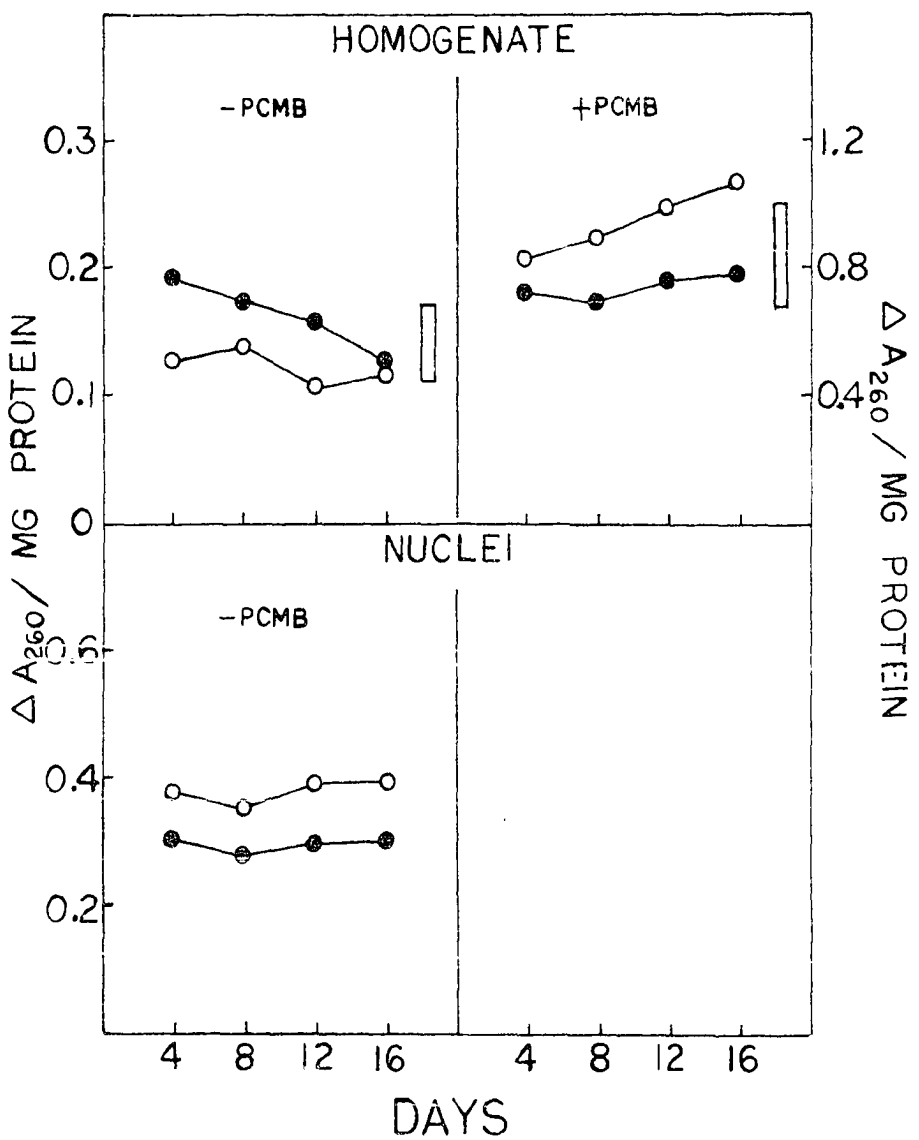
As shown in Figure 18, homogenate activity in the absence of PCMB was not significantly different at any time period due to amount of protein fed. When assayed in the presence of PCMB, the latent enzyme activity appeared to be higher in the animals receiving 5% casein, and the differences between dietary treatments widened with time but were not significantly different even by 12 and 16 days. The specific activity increase due to PCMB was greater than 4 fold, suggesting that over 75% of the enzymic activity in these preparations was latent.

Nuclear ribonuclease was assayed only in the absence of PCMB since no differences in rates were observed when assayed in the absence or presence of PCMB (results not shown) and since the RNAse inhibitor is localized in the cytosol fraction its presence in purified nuclei is minimal (Roth and Juster 1972).

The activity of nuclear preparations was higher in the

Figure 18

Ribonuclease activity in liver homogenates measured in the absence and presence of p-mercuribenzoate and in hepatic nuclear fractions measured only in the absence of the inhibitor. Liver homogenates and nuclear fractions of experiment II were prepared from animals that had received 5% or 20% casein-containing diets for the indicated time period. Enzyme assays contained 1.0 ml of a suitable dilution of liver homogenate in TKM buffer, 1.0 ml of substrate RNA (0.125%) in 0.10 M Tris-HCl, pH 7.5, and 1.0 ml of either H_2O or 1 mM p-chloro-mercuribenzoate (PCMB). The reactions (total volume = 3.0 ml) were stopped by the addition of an equal volume (3.0 ml) of 0.4 N $HClO_4$ containing 0.5% lanthanum acetate. After 10 minutes at 0 C, the precipitates were centrifuged at 2000 g for 10 minutes, and the supernatants were analyzed for absorbance at 260 nm. Nuclear fractions were assayed for ribonuclease activity in a similar fashion except that activities in the presence of PCMB were not measured (Bresnick, Sage, and Lanclos 1966). 5% casein o—o—o; 20% casein ●—●—●; the minimum significant difference ($P < 0.01$) is indicated by the open bar.


HOMOGENATE
-PCMB
+PCMB
NUCLEI
-PCMB
0.3
0.2
0.1
0
0.5
0.4
0.2
1.2
0.8
0.4
4
8
12
16
4
8
12
16
DAYS
Δ A260 / MG PROTEIN
Δ A260 / MG PROTEIN

case of animals fed 5% casein but the difference between any two treatment means was not significant. These trends, however, were opposite to those observed in homogenates when assays in the absence of PCMB were performed and are qualitatively similar to those for homogenates assayed in the presence of PCMB.

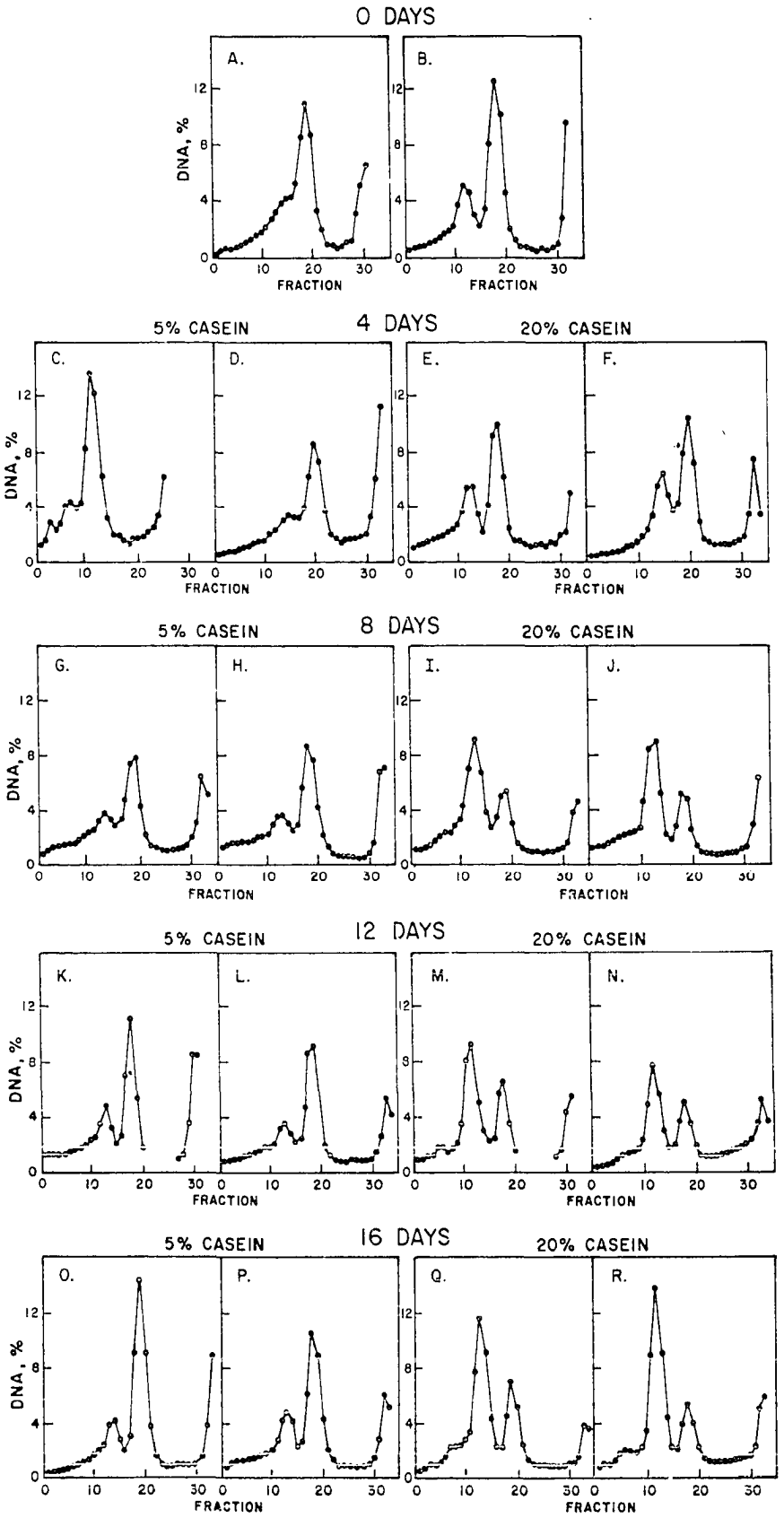
These studies suggested that protein deficiency did not result in major alterations in this enzyme nor was a marked compartmentalization of changes within the hepatic cell evident. The present findings are evaluated in the light of literature findings discussed below (see Discussion V,B.).

F. Ploidy of Hepatic Cellular Nuclei

Ploidy distribution studies were performed on liver nuclei of animals fed 5% or 20% casein for 0, 4, 8, 12, or 16 days. For each treatment group, two samples of nuclei (representing 4 pooled livers each) were each analyzed by sucrose gradient centrifugation. These results are shown in Figure 19 as a composite of all gradient analyses. The top center panels represent the zero time controls. The groups fed 5% casein are shown on the left and 20% casein on the right. Results of analyses of the zero time control groups (Panels A, B) show that the majority of the DNA was in the diploid

Figure 19

Sucrose density gradient sedimentation profiles of hepatic nuclei prepared from animals that had been fed 5% or 20% casein-containing diets for 0, 4, 8, 12, or 16 days. Nuclei were isolated and analyzed by density gradient centrifugation as described in MATERIALS AND METHODS and in the legends of Figures 3 and 4. Each sample represented the nuclear fraction obtained from homogenates of the pooled livers of four animals that had been fed 5% or 20% casein for the indicated period of time. For each time period and each level of dietary casein, the results of analysis of two separate samples are shown in adjacent panels. A total of 72 animals was employed for the eighteen gradient analyses shown. The DNA in each fraction is plotted as a percentage of the total DNA recovered from the gradient. Zero time controls: A,B; 5% casein: 4 days, C,D; 8 days, G,H; 12 days K,L; 16 days, O,P. 20% casein: 4 days, E,F; 8 days, I,J; 12 days, M,N; 16 days, Q,R.


0 DAYS
A.
B.
DNA, %
FRACTION
5% CASEIN
4 DAYS
20% CASEIN
C.
D.
E.
F.
8 DAYS
G.
H.
I.
J.
12 DAYS
K.
L.
M.
N.
16 DAYS
O.
P.
Q.
R.

class with only a small component of tetraploid nuclei seen as a shoulder ahead of the major peak of diploid nuclei (panel A) or as a small discrete peak (panel B) in the second sample.

Results of the two dietary groups after 4 days (panels C-F) on the feeding regiment demonstrate that nuclei of animals receiving 5% casein were distributed similarly to those of the zero time controls, whereas in those animals receiving 20% casein an increase in the tetraploid class was apparent. The differences are striking at 8 days (panels G-J). As at 4 days, animals fed 5% casein showed little changes from the zero time control group whereas the DNA of hepatic nuclei distributed predominantly as tetraploid nuclei in animals fed 20% casein. This increase in tetraploid nuclei continued through 12 days (panels K-N) and 16 days (panels M-R). In no instance did those animals restricted by 5% casein exhibit this appearance of tetraploid nuclei. The constancy of the relatively small amount of DNA in the tetraploid class of nuclei from animals receiving 5% casein is remarkable at all time periods (Panels C, D; G, H; K, L; O, P). In animals whose growth was not arrested by protein restriction, the time dependent increase in

tetraploidy is clear as well as the apparently reciprocal decrease in the frequency of diploid nuclei.

The sucrose gradient analyses were used to quantitate the relative amounts of DNA within each sedimentation class as well as the remaining regions of each gradient. Graphical representations of estimates of these distributions are illustrated in Figure 20. Within each gradient the recovered DNA was taken as 100%, and the percent of the recovered DNA in the diploid and tetraploid classes was estimated from the peak fraction and three fractions on either side. Since the peak diploid and tetraploid classes were separated by less than seven fractions in all gradients, the use of this convention caused overlap. Therefore, for the region between the peaks, one half of the recovered DNA in each overlapping fraction was considered diploid and one half tetraploid.

The relative distributions computed are illustrated in two ways. First, as shown in Figure 21, the distributions for all regions (I-V) of the gradients are illustrated. Each point is the average of two determinations, and the brackets denote the ranges of individual measurements. A substantial amount of recovered DNA did not sediment as diploid or tetraploid nuclei (zones III and IV). Heavy or aggregated nuclei

Figure 20

Estimation of the DNA distribution among the various sedimentation classes of hepatic nuclei. The percentages of recovered DNA (normalized to 100 percent) in the tetraploid zones (shaded areas) and in the diploid zones (stippled areas) were estimated from the sum of the DNA in the peak fraction and three fractions on either side. For fractions between the diploid and tetraploid peaks in which this convention could not be applied, the DNA was considered one half diploid and one half tetraploid. For the remainder of the gradient regions, the DNA recovered at the tube bottom is indicated as well as that recovered in zones heavier than tetraploid nuclei and as light or damaged nuclei near the meniscus. For the two gradients with missing data (fractions 20-28 in panels K and M), the estimate was based on the assumption of a smooth continuity in the DNA distribution in that region.

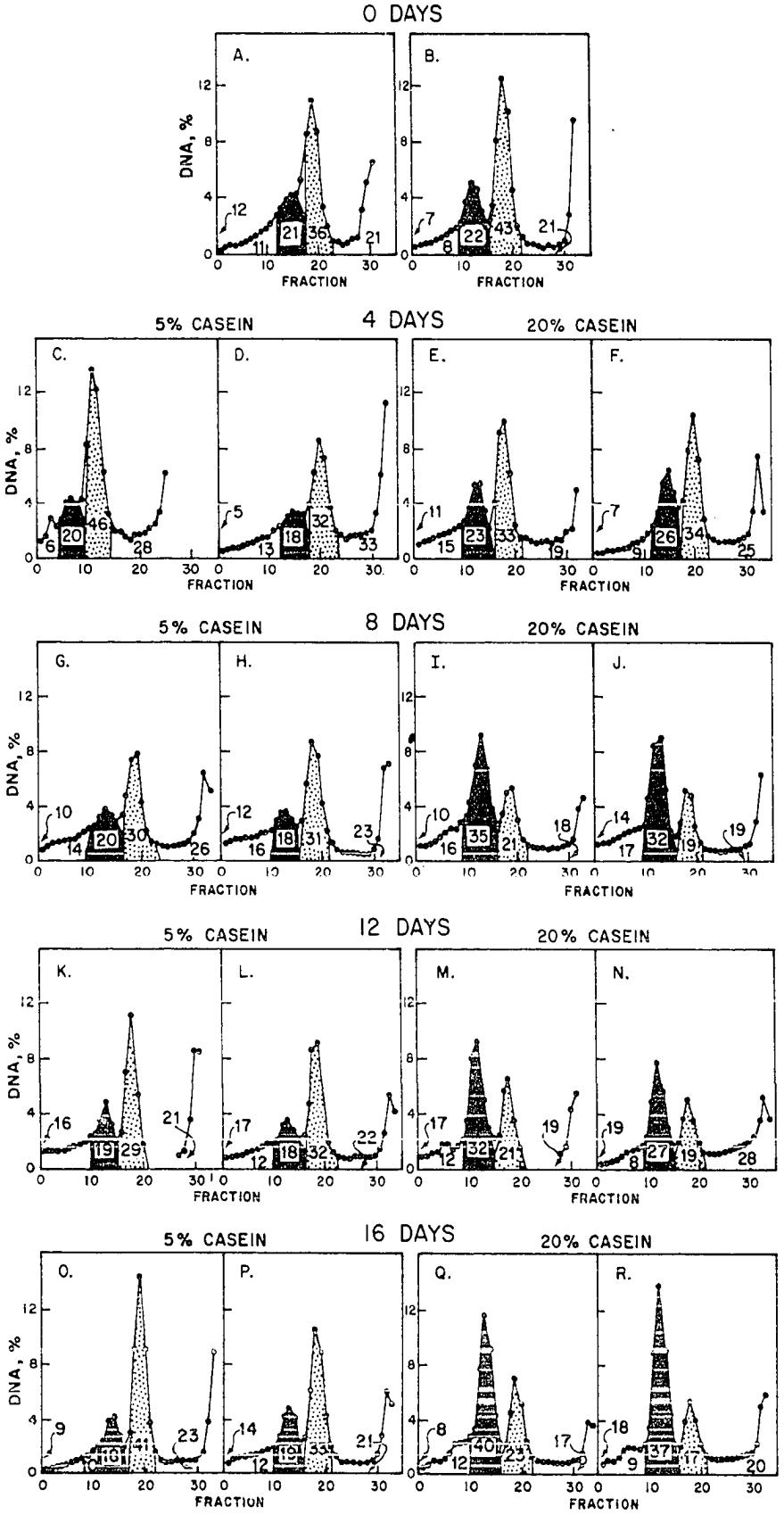
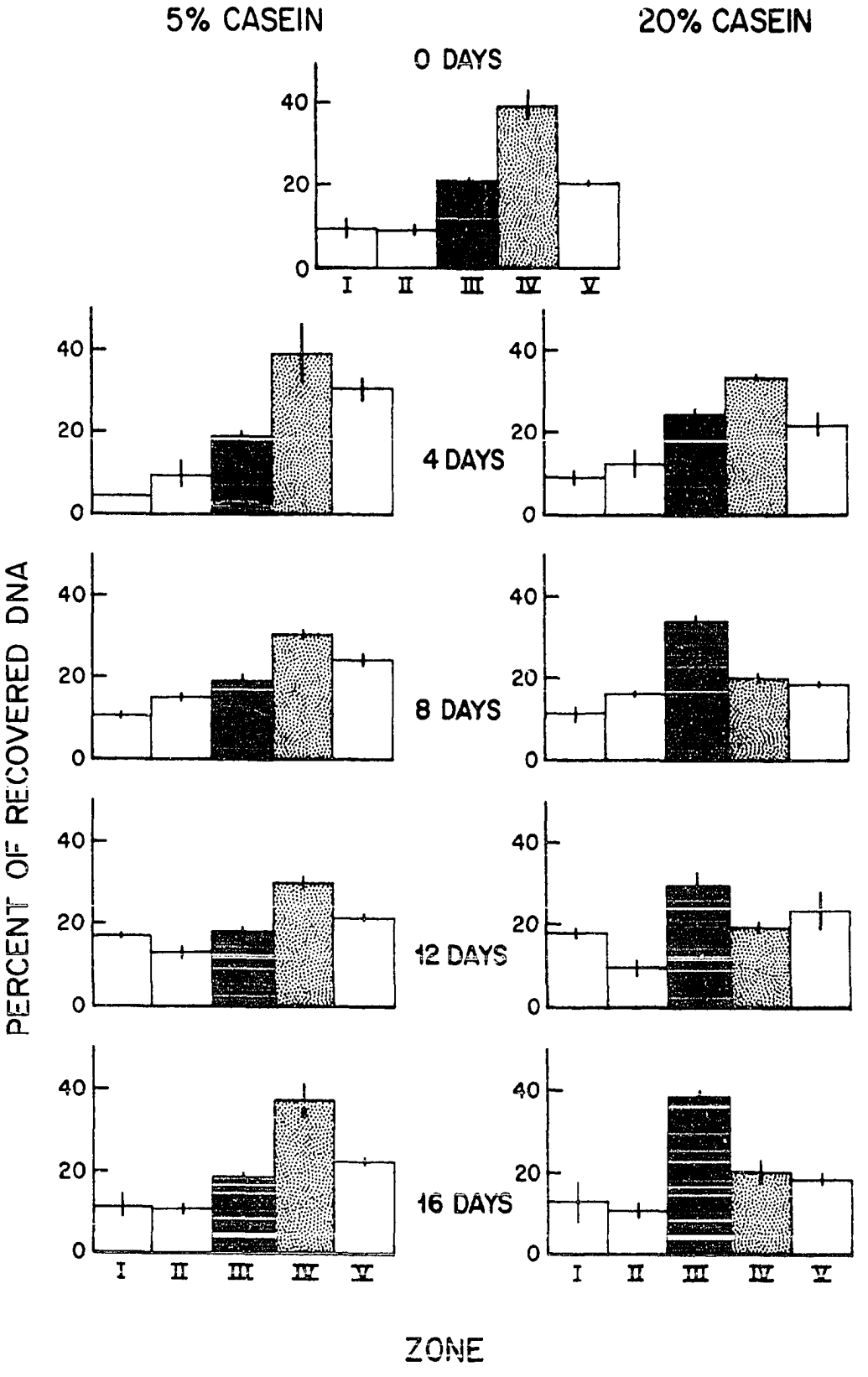

0 DAYS
A.
B.
DNA, %
FRACTION
5% CASEIN
4 DAYS
20% CASEIN
C.
D.
E.
F.
8 DAYS
G.
H.
I.
J.
12 DAYS
K.
L.
M.
N.
16 DAYS
O.
P.
Q.
R.

Figure 21

Relative distribution of DNA within all gradient sedimentation classes for animals fed 5% or 20% casein for 0, 4, 8, 12, or 16 days. The estimates described in Figure 20 for the fractional recoveries of DNA in each region (I-V) of the sucrose gradients have been plotted. Each value represents the average of two separate analyses indicated by the vertical bars. Zone I, pellet at bottom of tube; Zone II, nuclei larger than tetraploid; Zone III, tetraploid nuclei; Zone IV, diploid nuclei; Zone V, light or damaged nuclei.


5% CASEIN
20% CASEIN
0 DAYS
4 DAYS
8 DAYS
12 DAYS
16 DAYS
PERCENT OF RECOVERED DNA
ZONE
0
20
40
I
II
III
IV
V

(Zone I) and nuclei larger than tetraploid (Zone II) comprised between 12 and 30% of the recovered DNA, between 20 to 30% of the DNA was recovered as light or damaged nuclei (Zone V). Although recovery of DNA in zones I, II, and V may be, in part, artifacts of the procedure their relative amounts did not appear to vary between the two dietary treatment groups. Furthermore, Zones III (tetraploid nuclei) and IV (diploid nuclei) comprised the remainder and represented between 49 and 60% of the recovered DNA. The constancy of these two classes in animals fed 5% casein is in striking contrast to the changes shown for those fed 20% casein.

When classes I, II, and V were excluded and the relative frequencies of 2n and 4n nuclei considered as a binomial distribution, the data in Figure 22 were obtained. Expressed in this way the apparent changes in the relative proportions of tetraploid nuclei are more impressive. The data have been expressed as the fractional distribution of DNA (left ordinate) and as the fractional number of nuclei in the tetraploid class (right ordinate). Analyzed in these ways, the zero time controls contained only 35% of the nuclear DNA in the tetraploid class. In animals fed 20% casein, over 40% of the

DNA is in the tetraploid class by four days and the rise continues throughout the feeding regimen approaching 70% by the sixteenth day.

In animals fed 5% casein, apparent tetraploidy did not exceed 40% at any time period. Calculated as actual numbers of nuclei, values (shown on the right hand ordinate) suggest that only 20 to 25% of the nuclei may be tetraploid at weaning, that by 16 days on a diet supporting growth (20% casein) as much as 50% are tetraploid, and that under conditions of protein restriction these time-dependent changes do not occur.

Finally, the physiologic correlates of hepatic tetraploidy were assessed. When the estimate of relative tetraploidy shown in Figure 22 was plotted versus liver weight for all treatment groups, a correlation coefficient of 0.933 was obtained (Figure 23). Similar correlations were computed for body weight at autopsy ($r = 0.898$) and for body weights when the weight loss due to fasting was eliminated ($r = 0.912$). Using the index of tetraploidy shown in Figure 21, the correlation with liver weight was 0.906.

Figure 22

Alterations in the relative proportions of tetraploid nuclei in animals fed 20% casein and constancy of these proportions in animals fed 5% casein. The gradient analyses of Figure 20 were used to estimate the relative amount of DNA in each sedimentation class. Data are expressed both as the fraction of DNA and as the fractional number of nuclei, assuming the DNA content of tetraploid (4n) nuclei equals twice that of the diploid (2n) nuclei and using the sum of diploid plus tetraploid = 1.0. The vertical bars denote the ranges of duplicate analyses. Note the nonlinear scale for fractional number of nuclei. 5% casein, o—o—o; 20% casein, ●—●—●; zero time controls, ⊗.

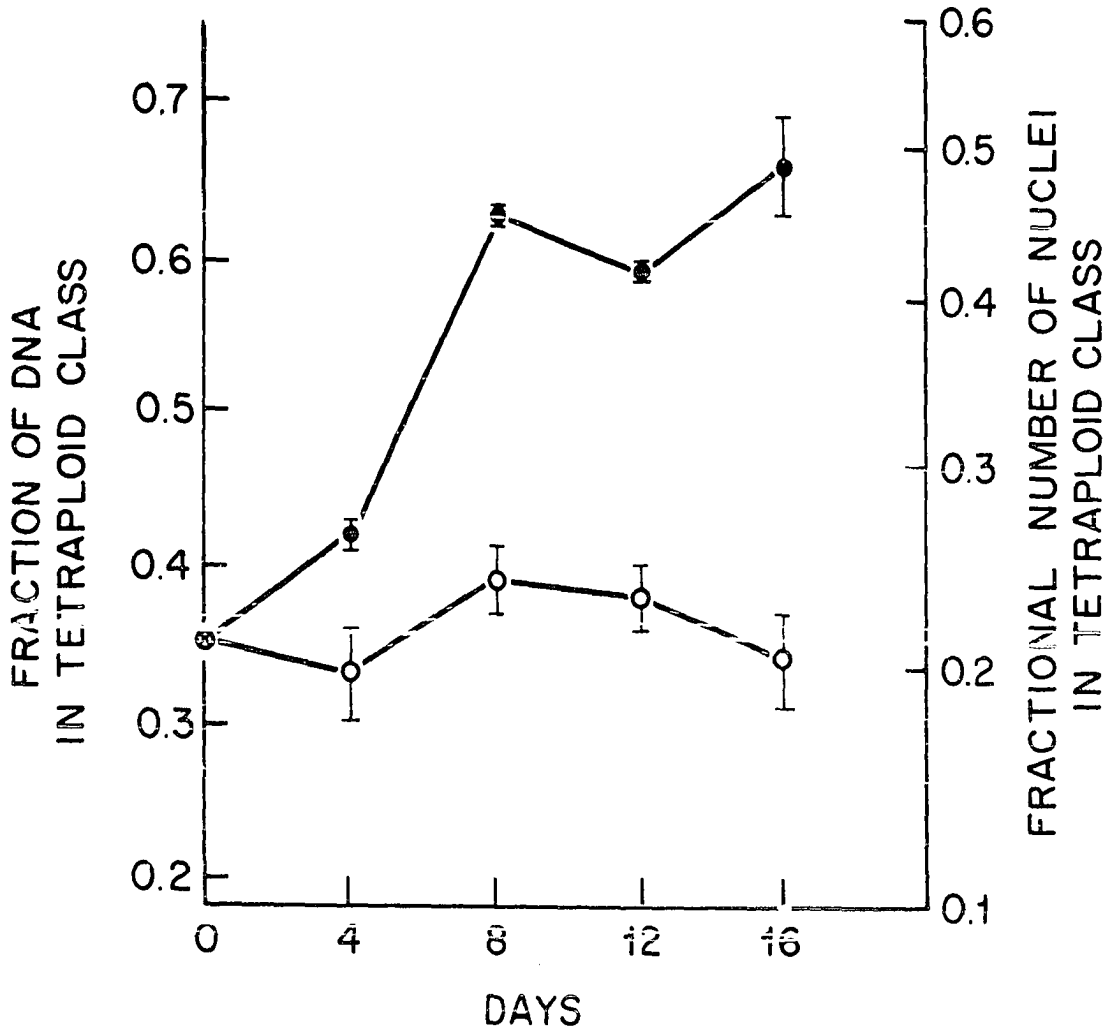
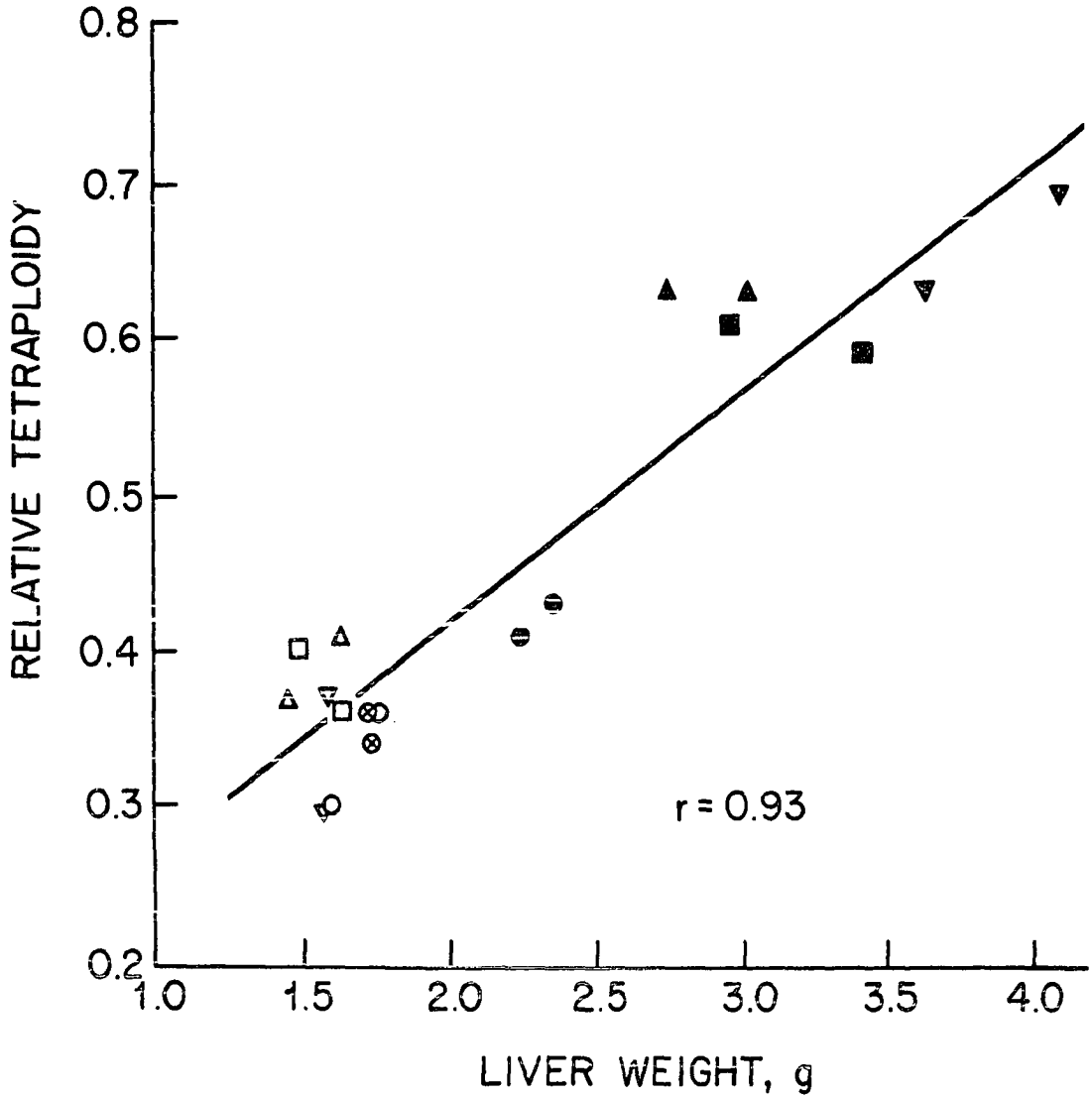

FRACTION OF DNA
IN TETRAPLOID CLASS
0.7
0.6
0.5
0.4
0.3
0.2
0
4
8
12
16
DAYS
0.6
0.5
0.4
0.3
0.2
0.1
FRACTIONAL NUMBER OF NUCLEI
IN TETRAPLOID CLASS

Figure 23

Correlation of relative tetraploidy with liver weight for all treatment groups of experiment III. The values for the fraction of DNA in the tetraploid class and the corresponding mean liver weights for the four animals represented by each point are shown for each treatment group. The correlation coefficient obtained by least squares analyses of the 18 determinations was 0.933. Zero time controls, ⊗; open symbols, 5% casein; closed symbols, 20% casein; 4 days, o or ●; 8 days △ or ▲ ; 12 days □ or ■ ; 16 days ▽ or ▼ .


0.8
0.7
0.6
0.5
0.4
0.3
0.2
RELATIVE TETRAPLOIDY
r = 0.93
1.0
1.5
2.0
2.5
3.0
3.5
4.0
LIVER WEIGHT, g

V. DISCUSSION

Two explanations for an elevated hepatic DNA concentration observed in protein deficiency are (i) a diminished cytoplasmic mass and volume, in the absence of a concomitant loss in nuclear DNA, resulting in a reduction in the tissue weight/DNA ratio (Winick and Noble 1966) and (ii) increased levels of DNA per cell, i.e., increases in nuclear ploidy (Umana 1965, 1967). It should be emphasized, hypothetically at least, that the two are not mutually exclusive, and the suggestion has been made that both are causally related to elevated DNA concentrations (Umana 1967). Although the present studies can address each of these two issues, the latter is the major focus. Present studies are consistent with the concept that protein deficiency *per se* does not directly increase or decrease ploidy. Rather, the present data indicate that the normal process of polyploidization is suppressed when the dietary protein intake is limited.

A. Suppression of Polyploidization During Protein Deficiency

Initial studies (described as experiment II) utilized the conventional chemical method of estimating nuclear ploidy and did not demonstrate a growth-associated pattern

of polyploidization which had been clearly documented by previous investigators (Naora 1957; Alfert and Geschwind 1958; Nadal and Zajdela 1966a). Furthermore, in experiment II differences attributable to protein restriction were not observed. However, the validity of those studies was questioned for the following reasons. First, a large variance existed in the data. Second, the hemocytometer method itself has limitations (Berkson et al. 1940); in addition clumping of nuclei was encountered. Third, a subsequent experiment yielded data in direct conflict with those of experiment II.

In experiment III, hepatic nuclei were separated into two major size classes on the basis of differences in their velocity of sedimentation. This technique had been employed previously to effect separation (at unit gravity) in 30 to 36 hours (Falzone, Barrows, and Yiengst 1962); however, the potential of the method was further exploited by the use of low speed centrifugation to achieve more rapid separations in conventional sucrose gradients (Albrecht 1968, 1969) and in zonal rotors (Johnston et al. 1968 a,b). Nuclei also have been separated into density classes by isopycnic centrifugation (Fisher, Holbrook, and Irvin 1963).

Although the nuclear classes obtained in the present studies were discrete on the basis of sedimentation properties, three other criteria were examined to confirm that the nuclei thus isolated were diploid and tetraploid. These criteria were (i) size studies based on microscopic measurements, (ii) size studies using an electronic particle counter, and (iii) chemical determination of the average DNA per nucleus within each sedimentation class.

Tetraploid nuclei separated by their differential rates of sedimentation have been shown previously to be larger on the basis of both microscopic measurements (Falzone, Barrows, and Yiengst 1962) and by size distribution studies using an electronic particle counter (Santen 1965). Furthermore, nuclei of the tetraploid class were found to contain approximately twice the DNA as the diploid class when enumerated by electronic particle counting. The present findings are also in agreement with the values reported by Falzone, Barrows, and Yiengst (1962) and Johnston et al. (1968a) and are confirmation of the validity of enumeration and size estimation by electronic particle counting for nuclei separated in this way (Santen 1965).

The demonstration in the present study that a distinctive

pattern of tetraploidization occurs during a narrow stage of postnatal growth in the rat is in agreement with the studies of others (Naora 1957; Alfert and Geschwind 1958; Nadal and Zajdela 1966a). The data are new in the sense that they have utilized recent methology to permit an independent quantitative assessment of hepatic tetraploidization.

In animals fed maintenance levels of dietary protein, the finding of a suppression of tetraploidization is in disagreement with previous studies suggesting that average hepatocyte ploidy in weanling rats is increased (Lecomte and de Smul 1952) or unaffected (Umana 1965) relative to controls fed adequate amounts of protein. The present data do not address the situation in older animals in which average ploidy has been purported to increase (Umana 1965, 1967; Ely and Ross 1951) or to be unchanged (Thomson et al. 1953) due to the protein content of the diet. However, suppression of tetraploid cell formation has previously been postulated to occur when weanling animals were fed 5% casein (Mariani et al. 1966) or diets otherwise limited in protein content (Nadal and Zajdela 1966b).

The present data clearly confirm the interpretations of Mariani and those of Nadal and Zajdela; furthermore, a

substantial extension of their findings may be inferred from the experiments reported herein. Specifically, the present data add significant insight concerning at least three important features of hepatic tetraploidy that were not shown by the studies of Mariani and coworkers (1966).

> First, velocity sedimentation techniques permitted a quantitative analysis of the relative diploidy and tetraploidy that accompanied protein deficiency; diploid and tetraploid nuclei were separated into discrete classes, and the relative numbers in each class were quantitated by DNA determination.
>
> Second, the effects of protein deficiency on suppressing tetraploidization could be detected within four days after the feeding of a low protein diet; the earliest time period studied by Mariani et al. (1966) was ten days. Thus, alterations in ploidy became manifest much sooner than previously recognized.
>
> Third, reduced protein intake resulted in suppression of hepatocyte polyploidization independently of detectable changes in the RNA and protein composition of hepatocyte nuclei. This clearly implies that the cytokinetic mechanisms responsible for tetraploid cell accumulation are selectively sensitive to protein deficiency. Simultaneously, measureable changes in total nuclear RNA and protein per unit DNA cannot be demonstrated.

Regarding the apparent discrepancy between the present study and the studies of Umana (Umana 1965; cf. Table I), reconciliation of the data must be attempted with some reservation. It should be emphasized that the disagreement with Umana's findings is not between results based upon ploidy measures by the hemocytometer method. In point of fact, the hemocytometer measurements of experiment II would have led to the same conclusion made by Umana, namely that average hepatocyte ploidy is unaffected by the feeding of low protein diets to weanling rats. It is conceivable that other factors (such as the nutritional environment prior to weaning or strain differences) may have contributed to an advanced state of tetraploidy prior to Umana's experiment. If advanced tetraploidy had occurred prior to feeding the low protein diet, differences within weanling animals due to diet could have been minimized or eliminated. Also, it should be noted parenthetically that the same argument could be made for the differences between experiments II and III in this report.

Conflicting published data notwithstanding, the fact that relative hepatic tetraploidy correlated highly ($r=0.93$)

with liver weight for all treatment groups in experiment III is additional evidence that it is a growth-associated event and that interruption of growth by restricting the intake of protein may be a major determinant in the process.

However, pair-feeding studies may be necessary to conclude whether protein or energy intake is the major determinant. It is clear from other studies that either caloric restriction or pituitary ablation can result in reduced polyploidy (Bass and Dunn 1957; Di Stefano and Diermeier 1959; Nadal and Zajdela 1966b). Furthermore, pair-feeding data suggest that the effects of pituitary ablation are not mediated through reduced food intake (Bass and Dunn 1957; Di Stefano and Diermeier 1959). The data of these two studies have been summarized previously (see II, A.2; Table II) in this text. Bass and Dunn studied animals that ranged between 123 and 132 grams initial body weight. Intact controls and hypophysectomized animals were fed ad libitum for 21 days. A third group of intact animals was pair-fed for 21 days with the hypophysectomized group. For the intact groups, the percentages of nuclei that were tetraploid were 74.2 and 77.4 (controls vs. paid-fed). After hypophysectomy for 21 days, the percentage

of tetraploid nuclei was 66.0 percent.

The data of Di Stefano and Diermeier were obtained on smaller animals (84 to 96 grams) after an experimental period of eight days. The percentages of nuclei that were tetraploid were as follows: intact ad libitum controls, 64.3; hypophysectomized, ad libitum 30.8; intact controls that were pair-fed with the hypophysectomized group, 57.4. Also, in addition, growth hormone given by injection did not reverse the effects of protein-restriction (Nadal and Zajdela 1966b) nor the effects of reduced food intake (Di Stefano and Diermeier 1959).

Although several aspects of the present studies purport to show a direct role of diet in the normal process of tetraploid cell formation, the evidence is not conclusive. First, the present studies do not distinguish between the limitation in dietary protein and increased carbohydrate content of the diet as factors influencing suppression of polyploid nuclei. Although these data provide strong presumptive evidence that the effects were causally related to the amount of dietary protein, an explicit answer could be obtained by varying the quality (in contrast to the quantity) of dietary protein at fixed intakes of carbohydrates. Second, the

relative contributions of reduced food intake must be considered before a specific role attributable to a limitation of the dietary amino acid supply can be assessed. In this regard, these studies lacked appropriate pair-fed controls. However, reduced caloric intake may have played a minor role in the mediation of these effects since the intakes of the two diets were shown to be very similar when computed per unit of body weight (Table IX). Third, the possibility should be considered that the eighteen hour starvation period prior to autopsy, as was routinely used in these experiments, could have altered or attenuated the development of polyploidy.

Finally, these studies do not permit a decision whether the effects observed were directly due to a limited supply of the amino acids or whether they occurred as a result of hormonal or other stimuli secondary to protein deprivation. On the basis of the known effects of food intake, protein intake, and growth hormone the possibility should be considered that the effects of protein restriction are mediated through decreased circulating levels of growth hormone. The concept is far from new, and "nutritional pseudohyphysectomy" was suggested by Mulinos and Pomerantz (1941) to explain the

similarities in severely undernourished and hypophysectomized animals. However, the indirect evidence from several studies cited below and the direct evidence from one study (Christensson et al. 1975) to be discussed makes this interpretation of the present findings an unlikely one.

The findings of Blazquez et al. (1974) and Strosser and Mialhe (1975) both suggested gradual elevations in serum rat growth hormone during the post-weaning period and up to 80 days of age. Although increased growth hormone may be a temporal correlate of tetraploidization, recent evidence for the rat suggested that circulating levels of the hormone were increased rather than decreased following 14 days on a non-protein diet (Christensson et al. 1975).

Furthermore, increased growth hormone levels have been associated with moderate protein deficiency in dogs (Heard et al. 1968) and with low protein diets fed to rabbits (Turner, Allen, and Munday 1974). In humans, increased plasma growth hormone levels are a component of the clinical picture in kwashiorkor (Pimstone et al. 1968; Beas et al. 1971; Samuel and Desphande 1972; Lunn et al. 1973; Raghuramulu and Jaya Rao 1974).

Second, tissue responsiveness to growth hormone should

be considered, and decreased tissue responsiveness to growth hormone has been postulated in kwashiorkor (Hadden and Rutishauser 1967). Insufficient data are presently available to determine whether the hepatic response may have been sufficiently altered in animals fed 5% protein to attenuate a growth hormone-mediated tetraploidization. In hypophysectomized rats (100 grams) fed a non-protein diet for 6 days, growth hormone increased the liver RNA/DNA ratio toward control values but combined growth hormone, hydrocortisone and triiodothyronine therapy were necessary to restore values to those of controls (Christensson et al. 1975). Finally, administration of biologically active growth hormone to rats fed maintenance amounts of protein ad libitum (Nadal and Zajdela 1966b) or restricted quantities of an otherwise adequate diet (Di Stefano and Diermeier 1959) did not increase tetraploid cell formation.

A direct role of dietary amino acids on DNA synthesis appears more likely. By definition, DNA synthesis is obligatory for tetraploidization; therefore it is not surprising that when protein deprivation is sufficient to block increases in total tissue DNA, tetraploidization is also prevented. However, precise mechanisms are unclear. Recent evidence

suggested that supply of dietary amino acids played a direct role in the regulation of hepatic DNA synthesis (Dallman and Manies 1973; Dallman Spirito, and Siimes 1974; Short et al. 1973). Within 12 hours after the initiation of a low (3.5%) protein diet to 28 day old rats, DNA synthesis (estimated by ^{3}H-thymidine incorporation) and thymidine kinase (thought to be rate limiting in DNA synthesis) were depressed over 50% (Dallman and Manies 1973). Furthermore, a food-dependent peak in DNA synthesis occurred within 12 hours after feeding a normal diet to control animals and the food-dependent peak was much reduced in animals that had been fed a low protein (3.5%) diet for 31 to 32 days (Dallman, Spirito, and Siimes 1974).

Finally, DNA synthesis in the liver was stimulated more than 10-fold following the shift from either a protein-free or 6% casein diet to a diet containing 24% casein (Short et al. 1973). Furthermore, when selected mixtures of four l-amino acids were added at a level of 6% to the protein-free diet prior to the shift of 24% casein, one mixture (methionine, phenylalanine, serine, and tryptophan) totally inhibited the subsequent stimulation in DNA synthesis whereas a second mixture (cystine, methionine, serine, and threonine) was without effect.

B. Maintenance of Nuclear RNA and Protein During Protein Deficiency

Some hepatic constituents were found to change in concentrations in liver tissue in response to restricted protein intake. Concentrations of DNA were slightly elevated or unaffected, protein concentrations were decreased, and RNA closely paralleled overall hepatic weight and its concentration was not altered. In adult animals subjected to comparable or more extreme degrees of protein deficiency, there are rapid and marked losses in cellular RNA (Campbell and Kosterlitz 1950; Munro, Naismith and Wikramanayake 1953; Munro and Clark 1960) and parallel diminutions in hepatic protein (Allison and Wannemacher 1965; Lal, Agarwal, and Shankar 1975). In the present studies, the ratios of both RNA/DNA and protein/DNA of homogenates did decrease in a temporal fashion during protein restriction, whereas these ratios in control animals were constant between 4 and 16 days.

In contrast to whole cell homogenates, a change in RNA/DNA and protein/DNA ratios of the nuclear fractions did not occur. The fact that RNA/DNA and protein/DNA ratios of purified nuclear fractions approximated 0.2 and 2, respectively

for all treatment groups suggested a marked resistance of this cell organelle to changes in protein deficiency.

These findings are in agreement with those of Thomson et al. (1953) and of Munro, Waddington, and Begg (1965) but require further consideration in the light of two subsequent studies (Wannemacher, Wannemacher and Yatvin 1971; Andersson and von der Decken 1975). The data of Wannemacher and coworkers were expressed on a "per nucleus" basis and comparisons were made on weanling (23 day old) animals that were fed 6% or 18% casein for 18 days. Both RNA/nucleus and protein/nucleus in animals fed 18% casein were elevated compared to those fed 6% casein. If, due to tetraploidization, elevations in average DNA/nucleus also occurred only in animals fed 18% casein, their data may reflect suppression of tetraploidization in animals fed 6% casein and are compatible with the present study.

The data of Andersson and von der Decken (1975) were expressed per unit DNA, and showed that liver nuclei, isolated from 70 to 80 gram animals after a feeding experiment of six days duration, contained 11.1 or 24.6 mg protein/mg DNA for animals that were fed 3% or 20% protein. Both of those values are extraordinarily high when compared with those obtained by others using the same method of nuclear isolation. It was noted

previously that protein/DNA ratios of 1.8 to 2.6 have been reported for purified hepatic nuclei (Kashnig and Kasper 1969; Tata 1974); in the present studies all values were between 1.88 and 2.38 mg protein/mg DNA.

The evidence at hand overwhelmingly and strongly favors the stability of nuclear RNA and protein under conditions in which moderate or marked alterations in extranuclear amounts of these constituents occur.

Studies of alkaline ribonuclease in the nuclear fraction were performed in order to determine whether differential changes in RNA degradation might occur for nuclear fractions and whole homogenates. The small fraction of homogenate activity found in the nuclear fraction is in agreement with studies of Rosso, Nelson, and Winick (1973). However, the issue of nutritional regulation by the quantity of protein in the diet is complicated by the fact that the enzyme exhibits extensive activation when assays are performed in the presence of sulfhdryl antagonists, a latency due to the presence of a naturally occurring protein inhibitor of the enzyme (Roth 1956). As a consequence, nutritional control conceivably could be mediated through the amount of enzyme or by the amount of inhibitor protein or both.

Measurements of both "free" and "total" enzyme activity are imperative for an adequate interpretation.

The apparent lack of effect of feeding 5% casein for up to 16 days on nucleus-associated ribonuclease as well as on homogenate activities (without and with PCMB) adds to the existing disagreement in the literature on this subject. For example, more extreme degrees of protein deprivation have produced increased specific activities by 21 days in the "free" activity in young (50 gram) rats (Leutskii and Garmatii 1964) as well as more mature ones (Girija, Pradhan, and Sreenivasan 1965). Others have been unable to confirm these effects when protein-free diets were fed for up to 22 days (Matsuo et al. 1966).

"Total" ribonuclease, e.g., the activity in the presence of PCMB, reportedly was unaffected in growing as well as adult animals when protein-free diets were fed for 15 or 30 days (Benedetti et al. 1966 a,b), although experimental malnutrition in infant rats has provoked marked decreases in this parameter (Rosso and Winick 1975).

The most convincing evidence for any regulation by dietary protein of this enzyme-inhibitor system has come from studies showing reduced levels of the inhibitor in

protein deficiency. Both by direct assay of the ability of cytosol to inhibit a standard enzyme assay (Girija, Pradhan, and Sreenivasan 1965) and by the extent of activation by PCMB (Quirin-Stricker, Gross, and Mandel 1968), experiments have ascribed a reduction in the ribonuclease inhibitor attributable to protein deprivation. Similar findings have been confirmed for the malnourished infant rat (Rosso and Winick 1975).

Although the present studies were unable to demonstrate a significant effect on the activity of the enzyme, variables such as the degree of severity and duration of the deficiency state as well as energy intake should be kept in mind.

C. Functional Significance of Tetraploidization in Postnatal Growth and of its Suppression in Protein Deficiency

The functional significance of polyploidy is far from clear. Its occurrence in the natural ontogeny in the liver of the rat, mouse (Gerhard, Schultze, and Maurer 1971), and man (Guimaraes 1971) is in contrast to an absence of this feature in the guinea pig (Carriere 1969). Although it primarily occurs in liver, evidence suggests that tetraploidy in other tissues occurs in normal as well as pathologic states.

Polyploidy has been shown to occur to some degree in the rat thyroid, adrenal, and pituitary (Racadot 1964). Certain neurons are polyploid (Novakova, Sandritter, and Schlueter 1970) and recent studies suggest that neuronal nuclei are tetraploid and glial nuclei are diploid (Bregnard, Knüsel, and Kuenzle 1975). Hypertrophied ventricular myocardium has been shown to contain larger proportions of tetraploid nuclei than normal myocardium (Pfitzer and Capurso 1970).

At least two pathologies of nutritional origin have been described. The toxic agent in soy protein (lysinoalanine) has been causally related to polyploidy in kidney

cortical tissue (Reyniers, Woodard, and Alvarez 1974). Excess dietary methionine induces polyploidy in the rat pancreas although it does not exacerbate or attenuate the existing polyploid state of liver (Bourdel, Girard-Globa, and Forestier 1971).

Several lines of evidence suggest that some degree of functional significance might be ascribed to tetraploidy in regard to the capacity for protein synthesis or cellular growth. For example, Johnston et al. (1968b) described biochemical heterogeneity in nuclear classes resolved in the zonal centrifuge. The RNA polymerase activity of tetraploid nuclei was over twice that observed in diploid nuclei. It has also been suggested that tetraploid nuclei play a more important role in liver regeneration than do diploid nuclei (Fugita et al. 1974). In addition, Painter and Biesele (1966) have proposed an increased capacity for polyribosome formation in polyploidy, and secretory activity has been correlated with cellular ploidy in invertebrates (Floyd and Swartz 1969).

Whatever improvements or alterations in functional capacity may be conferred in the normal state by increased ploidy, the suppression of the process during protein

deficiency is consistent with the concept of a maintenance of indispensable cellular function without increases in cell size as was suggested by Dallman and Manies (1973). The volume-ploidy relationship would suggest that a single tetraploid cell can be considered approximately equivalent to two mononuclear diploid cells (Epstein 1967).

D. Adequacy of Methodology

Two crucial aspects of these studies from a methodologic standpoint were (i) the nuclear isolations and (ii) the density gradient separations of diploid and tetraploid nuclei. The current evidence suggests these are proven and well established procedures.

1. Nuclear isolation

In a recent review (Tata 1974), aspects of currently popular methods of nuclear isolation were discussed. Of the two major methods described in that review, one was utilized in the present work, namely that of Blobel and Potter (1966). Its ability to afford nuclei of exceedingly high purity is attested to by the fact that it has been employed in studies on the enzymology of the nuclear membrane (Kashnig and Kasper 1969; Gunderson and Nordlie 1975) in

which minimization of cytoplasmic contamination is absolutely essential.

The data of the present report are compatible with acceptable purity based on the criteria of RNA/DNA and protein/DNA ratios. These ratios, which approximated 0.2 and 2.0 respectively, are in close agreement with the published data from other laboratories (Blobel and Potter 1966; Kashnig and Kasper 1969; Tata 1974).

In addition to purity, the other aspect is whether the isolated nuclei are representative of the total nuclear population of the tissue. In tissues such as the pancreas, the method has been reported to provide nuclear fractions representative of the whole homogenate (Bourdel, Girard-Globa, and Forestier 1971). For liver, however, evidence suggests that the use of the method is accompanied by some differential destruction between normal and regenerating liver cells as well as a selectivity between hepatocyte and stromal cells. Lewan (1971) had adduced that a selective fragmentation of newly formed hepatocytes occurred during homogenization. Lewan's study was based on a comparison of the number of nuclei per unit volume of liver. Measurements were made for both liver sections and homogenates

prepared at intervals up to 140 hours post-hepatectomy. No differences between sections and homogenates occurred until after mitosis (i.e. after 24 hours). Thereafter (between 24 and 72 hours) the number of nuclei per unit volume increased when measured in sections but decreased when measured in homogenates. Such an observation is more important in studies of regenerating liver than in studies of normal tissue. On the other hand, hepatic nuclear fractions prepared by this method have been shown to be enriched in parenchymal relative to stromal nuclei and therefore are representative only of the hepatocyte population and not the total tissue (Bushnell, Whittle, and Potter 1969). Such a selectivity would not appear to be a major limitation in the present studies, since tetraploidy is limited to the parenchymal cells (Falzone, Barrows, and Yiengst 1962).

Additional support for the Blobel and Potter (1966) method may be found in its use in studies on the localization of DNA polymerase (Bollum 1975). However, the fact that more than 50% of the total DNA polymerase has been found in the cytoplasmic fraction of cell homogenates is paradoxical, and has been interpreted as evidence that " ... all currently 'acceptable' cell fractionation procedures may be inadequate". (Bollum 1975).

Finally, Tata (1974) recommended that homogenates should be filtered through 120 mesh nylon prior to the nuclear isolation. This filtration would be quite similar to the cheesecloth (grade #120) filtration adopted for the present study in experiment III. Preparations of hepatocyte nuclei made in Tata's laboratory were also examined with respect to recovery. Recoveries (as percent of total tissue DNA) were 70.4 ± 4.9 and 74.8 ± 4.3 percent for the Widnell and Tata (1964) and Blobel and Potter (1966) methods respectively.

In summary, the available evidence suggests that the Blobel and Potter (1966) method of nuclear isolation affords nuclei of equal or greater purity than that of other methods and that the nuclei thus isolated reflect the parenchymal cell population but not the total tissue.

2. Density gradient separation of diploid and tetraploid nuclei

The potential value of the velocity of sedimentation technique in separations is not only useful for nuclei but for various cell types of differing sizes or densities. The theoretical and practical aspects of cell separation by velocity of sedimentation is the subject of a recent review (Harwood 1974). In addition, the principle has been utilized

recently to provide the most homogeneous preparations of rat testis cell types heretofore possible (Platz et al. 1975) and also has been employed to separate bone marrow erythroid cells (Denton and Arnstein 1973) and to purify Purkinje neurons (Cohen, Mares, and Lodin 1973). Rat liver nucleoli may be resolved into different classes based on their sedimentation properties (Johnston et al. 1969).

Hepatic nuclei have now been separated into diploid and tetraploid classes in several laboratories (Johnston et al. 1968 a,b; Albrecht 1968; Gonzales-Mujica and Mathias 1973). In addition, the method has been used to study distribution of diploid and tetraploid nuclei in hepatomas (Albrecht 1969). In all of these studies, the two major classes of nuclei have been identified as diploid and tetraploid nuclei by one or more criteria. Nuclear size and DNA contents have been shown to be very near expected ratios for the two classes.

The relationship of ploidy to the nutritional state has not been examined by sedimentation techniques. However, a relationship to age and body weight has been established using zonal centrifugation (Johnston et al. 1968a; Gonzales-Mujica and Mathias 1973). According to Johnston et al. (1968a) zonal centrifugation resolves diploid and tetraploid

parenchymal nuclei and partially resolves stromal and parenchymal diploid nuclei within the same gradient. They reported that livers of newborn rats contain very few tetraploid nuclei which do not become distinguishable until the fifth week after birth (at 100 grams body weight). In their study, data were obtained at eight time periods between birth and maturity in the postnatal development of Norwegian Hooded rats (sex not stated). Zonal profiles of hepatic nuclei, isolated by the Widnell and Tata (1964) method, were reported for newborns (4.5 grams) and for animals at ages 3 weeks (25 grams), 4 weeks (50 grams), 5 weeks (100 grams), 7 weeks (150 grams), 13 weeks (200 grams), 6 months (300 grams) and 1 year (350 grams). A progressive increase in tetraploidy was noted between the fifth week (at 100 grams body weight) and advanced maturity (at 350 grams body weight).

Subsequently, Gonzalez-Mujica and Mathias (1973) reported zonal profiles for rat liver nuclei obtained from male rats of the Wistar Porton strain at different ages during postnatal development. Animals initially weighing 50 grams were employed, and zonal centrifugation data was obtained after 3, 6, and 8 weeks of normal growth. Body weight at these time periods had increased to 100, 170, and 250 grams,

respectively. Quantitative data on tetraploidy were not reported, although zonal profiles for nuclear fractions indicated that after three weeks of growth (at 100 grams body weight) tetraploid nuclei began to appear and progressively increased with time at six and eight weeks of growth. They also noted that only minor changes in relative tetraploidy occurred thereafter, even up to body weight increases of 300 grams.

The separation of nuclear classes by velocity of sedimentation has been extended to nervous system tissue (Austoker, Cos, and Mathias 1972; Cohen, Mares, and Lodin 1973) in which the issue of ploidy states is still controversial.

In summary, it would appear that gradient sedimentation is useful in the separation of various cell types and of the major nuclear classes. The method also could be exceedingly valuable in future assessment of ploidy states in a variety of nutritional pathologies and deficiencies as well as in normal tissue growth and differentiation.

VI. SUMMARY AND CONCLUSIONS

The extent to which a deficiency state with respect to dietary protein leads to altered biochemical and cytological features of the cell nucleus has been investigated in the past, but published data present conflicting biochemical and cytological observations. Variables for which data do not agree are changes in the RNA and protein composition and ploidy of the nucleus due to protein deficiency. In the present work the data suggest that the major effect of protein deficiency on hepatocyte nuclei in weanling rats is manifested as a suppression of the normal process of tetraploidization rather than as altered nuclear composition.

Nuclear RNA and protein (per mg DNA) were unaltered in protein-deficient animals, in contrast to homogenate RNA and protein (per mg DNA). Although nuclear alkaline ribonuclease (per mg of nuclear protein) was not affected, this finding alone is of limited significance because changes in the specific activity of this enzyme in whole cell homogenates did not occur.

Nuclear ploidy in protein deficiency was evaluated by measuring two major nuclear size classes, i.e., diploid (2n) and tetraploid (4n). Tetraploidization was then examined in

animals fed 5% or 20% casein for 4, 8, 12, or 16 days. Considering only two nuclear classes 20 to 25 percent of the hepatic nuclei were tetraploid in weanling rats, but after 16 days of near-optimal growth on a diet providing 20% casein, more than 50% were tetraploid. Formation of tetraploid nuclei was suppressed in animals fed 5% casein, and deviations from the initial ploidy distribution at weaning were minimal over the time periods studied. The fact that tetraploidy correlated highly ($r = 0.93$) with liver weight was interpreted as additional evidence that the process is growth-dependent and age-independent if growth is restricted.

A suppression of tetraploidization in protein restriction is consistent with studies from other laboratories suggesting that DNA synthesis is rapidly and markedly diminished in the absence of an adequate protein intake. Also, the present findings are compatible with the concept that the suppression of tetraploid cell formation is the result of a direct effect on DNA synthesis.

The functional significance of tetraploidy during growth is far from clear. However, several possibilities may be inferred from studies on polyploidy under other

conditions. For example, diminished RNA polymerase activity, decreased capacity for polyribosome formation, or otherwise diminished cellular activity conceivably could be associated with retention of the diploid state. Because cell size is proportional to the ploidy state, maintenance of diploidy during limited dietary protein intake may represent maintenance of an indispensable cellular function at the expense of cell size.

In conclusion, the assessment of ploidy by sucrose gradient sedimentation analyses may be valuable data in studies of nutritional pathologies or deficiencies. Further, reexamination of hepatic ploidy in adult animals is recommended because it is widely accepted that average cellular ploidy is unaffected by a variety of dietary conditions despite claims to the contrary. However, due to the relationship of ploidy to age, it is possible that dietary effects can only be demonstrated during growth.

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APPENDIX

Table XI. Summary of analysis of variance for data on hepatic DNA concentration (mg/g liver)[a] in experiment II.

Source	d.f.	S.S.	M.S.	F
Treatments	7	4.0992	0.5856	10.70**
A (level of protein)	1	2.9540	2.9540	54.00**
B (days of feeding)	3	0.1955	0.0652	1.19
AB	3	0.9497	0.3166	5.79**
Error	16	0.8754	0.0547	
Total	23	4.9746		

[a]Mean values are plotted in figure 11.

**$P < 0.01$

Table XII. Summary of analysis of variance for data on hepatic DNA concentration (mg/g liver)[a] in experiment III.

Source	d.f.	S.S.	M.S.	F
Treatments	7	1.9655	0.2808	1.52
A (level of protein)	1	1.1782	1.1782	6.36*+
B (days of feeding)	3	0.7082	0.2361	1.27
AB	3	0.0791	0.0264	0.14
Error	23[b]	4.2425	0.1845	
Total	30[b]	6.2080		

[a]Mean values are plotted in figure 11.

*+ $P < 0.025$

[b]One degree of freedom subtracted due to a single missing value in the data. An estimate of the missing value was computed according to a formula for a randomized complete-block design as described in Steel and Torrie (1960).

XIII. Summary of analysis of variance for data on hepatic RNA concentration (mg/g liver)[a] in experiment II.

Source	d.f.	S.S.	M.S.	F
Treatments	7	3.9476	0.5639	2.31
A (level of protein)	1	0.1093	0.1093	0.45
B (days of feeding)	3	2.2667	0.7556	3.10
AB	3	1.5716	0.5239	2.15
Error	16	3.9059	0.2441	
Total	23	7.8535		

[a]Mean values are plotted in figure 12.

Table XIV. Summary of analysis of variance for data on hepatic RNA concentration (mg/g liver)[a] in experiment III.

Source	d.f.	S.S.	M.S.	F
Treatments	7	1.7263	0.2466	1.87
A (level of protein)	1	0.0528	0.0528	0.40
B (days of feeding)	3	1.5091	0.5030	3.83
AB	3	0.1644	0.0548	0.42
Error	23[b]	3.0172	0.1312	
Total	30[b]	4.7435		

[a]Mean values are plotted in figure 12.

[b]One degree of freedom subtracted due to a single missing value in the data. An estimate of the missing value was computed according to a formula for a randomized complete-block design as described in Steel and Torrie (1960).

Table XV. Summary of analysis of variance for data on hepatic protein concentration (mg/g liver)[a] in experiment II.

Source	d.f.	S.S.	M.S.	F
Treatments	7	6453	921.9	9.05**
A (level of protein)	1	5017	5017.0	49.24**
B (days of feeding)	3	939	313.0	3.07
AB	3	497	165.7	1.63
Error	16	1630	101.9	
Total	23	8083		

[a]Mean values are plotted in figure 13.

**$P < 0.01$

Table XVI. Summary of analysis of variance for data on hepatic protein concentration (mg/g liver)[a] in experiment III.

Source	d.f.	S.S.	M.S.	F
Treatments	7	1047	149.6	2.69
A (level of protein)	1	936	936.0	16.80**
B (days of feeding)	3	16	5.3	0.10
AB	3	95	31.7	0.60
Error	23[b]	1282	55.7	
Total	30[b]	2329		

[a]Mean values are plotted in figure 13.

**$P < 0.01$

[b]One degree of freedom subtracted due to a single missing value in the data. An estimate of the missing value was computed according to a formula for a randomized complete-block design as described in Steel and Torrie (1960).