THE EFFECT OF DIETARY RETINYL PALMITATE AND RETINOIC ACID ON ORNITHINE DECARBOXYLASE ACTIVITY AND POLYAMINE CONCENTRATION IN RAT TESTES

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PREFACE

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CHAPTER I

INTRODUCTION

Vitamin A is involved in cell proliferation and differentiation as noted in the earlier report by Wohlbach and Howe (1925). Likewise, elevated polyamine levels and increased activity of ornithine decarboxylase (ODC), the rate-limiting enzyme in the polyamine biosynthetic pathway, are associated with growth in various tissues (Russell and Snyder, 1968; Jänne and Raina, 1968; Jänne et al., 1978). To date, the respective mechanisms of action have not been elucidated, but the related functions suggest a possible link between vitamin A and the polyamine biosynthetic pathway. Except for the function of retinal in the visual cycle (Wald, 1935), the mode of action of vitamin A has not been determined. Considerable effort has been expended in the search for a possible common denominator for vitamin A, ODC and the polyamines.

Function of Vitamin A

Vitamin A, or the retinoids (a term encompassing all forms of vitamin A), play a critical role in numerous known biological functions. First of all, vitamin A is necessary for normal growth and cell differentiation (Wohlbach and Howe, 1925). Second, it is crucial as a precursor of the visual pigments (Wald, 1935). Third, vitamin A is needed to maintain healthy epithelial tissues (Wohlbach and Howe, 1925). In the absence of this fat-soluble vitamin, epithelia undergo

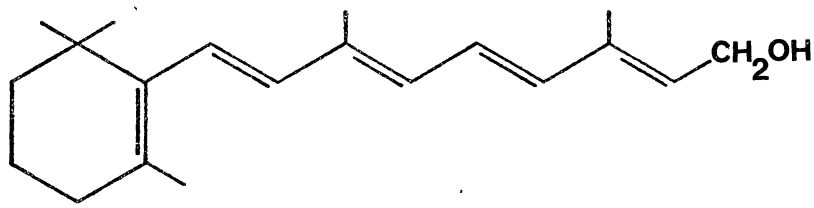
keratinization and fail to differentiate properly. Finally, vitamin A is required for normal reproductive functioning. In vitamin A-deficient female rats, resorption of the fetus results after mating (Howell et al., 1964). In males, vitamin A deficiency is characterized by testicular edema, degeneration (Wohlbach and Howe, 1925) and cessation of spermatogenesis (Howell et al., 1963).

The three major forms of vitamin A are retinol, retinal and retinoic acid (see Figure 1). Retinol can be utilized for growth, maintenance of epithelia and reproduction. It can also be oxidized to retinal to participate in the visual cycle. However, once retinol is oxidized to retinoic acid, it cannot be reduced back to retinol in vivo (Dowling and Wald, 1960; Olson et al., 1976). Although retinoic acid can function in specific vitamin A roles such as growth and differentiation, it can neither substitute for nor be reduced to retinal. Furthermore, it cannot replace retinol for maintenance of normal reproductive tissue. As a result, rats on vitamin A-deficient diets supplemented with retinoic acid grow well (Arens and van Dorp, 1946) but blindness gradually ensues (Dowling and Wald, 1960) and reproduction ceases (Thompson et al., 1964).

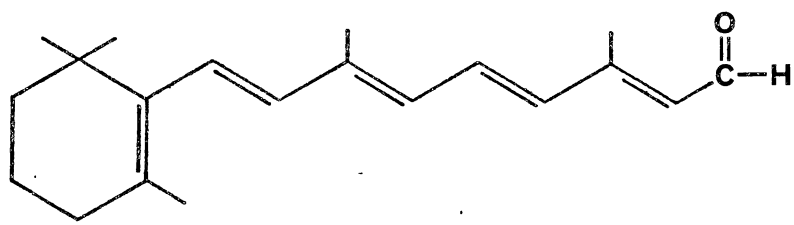
Vitamin A in Rat Testes

It has been over half a century since Wohlbach and Howe (1925) published their monumental work describing in detail the effect of vitamin A deficiency on various tissues of the rat. They confirmed what previous investigators had reported; that general growth retardation and blindness occur in vitamin A-deficient rats. More specifically, in place of normal epithelia, they found stratified

RETINOL



RETINAL



RETINOIC ACID

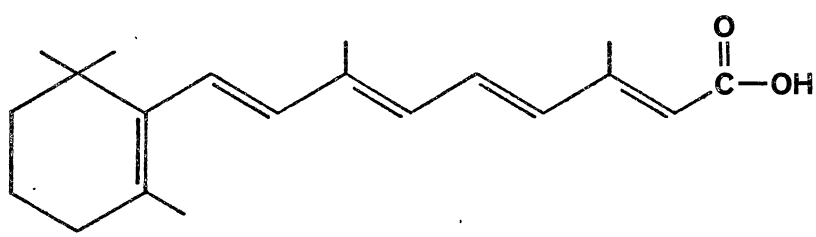


Figure 1. Vitamin A Structures

squamous keratinizing epithelium. In addition, atrophy was noted in all glands prior to keratinization. Infection was a common finding among many of the organ systems. Of particular interest to the present study is the effect of retinol deficiency on the testes. Wohlbach and Howe (1925) gave a detailed description of the consequences of prolonged deficiency in testicular tissue.

> Edema of the testes during atrophy of the seminiferous tubules reaches extreme degrees. The liquid lies outside the basement membrane, the connective tissue between the tubules does not seem to be permeated by liquid, so that the appearance in sections is that of tubules floating in a liquid medium. The liquid is evidently rich in protein material as it furnishes a heavy precipitate of coarse hyaline globules. The atrophy of the tubules when complete leaves apparently only cells derived from the sustentacular cells. The cells frequently have two to four nuclei. Early stages in the atrophy show spermatids with scattered chromatin particles and a structureless layer of material probably derived from the cytoplasm of these cells (p. 769).

Studies have been conducted to determine the distribution of vitamin A in the testes (Ahluwalia et al., 1975). Incorporation of labeled retinyl acetate and retinoic acid were measured in testicular tissue of rats and humans. Ahluwalia and his group reported that the vast majority of retinol was found in the seminiferous tubules, primarily in the cytosol. Of the cellular components, the Sertoli cells contained the most retinyl acetate, although the label was also detected in Golgi apparatus and endoplasmic reticulum. Retinol was not present in the interstitium. As for retinoic acid, no radioactivity was detected in the testis following injections. The reason for this is unclear. Ahluwalia et al. (1975) contend that this form of the vitamin may not be stored in the testis or may be rapidly oxidized. In contrast, McGuire et al. (1981) have recently confirmed

via autoradiographic studies that the interstitium is, in fact, the entry point for vitamin A to the testes. Moreover, one hour after injection with [^{125}I]retinol-binding protein, the radioactivity was still almost entirely restricted to the interstitial cells. The mechanism by which retinol crosses into the Sertoli cells to support spermatogenesis and the general epithelia is unknown.

Until recently, retinoic acid was thought to be without function in the testes despite data demonstrating the presence of cellular retinoic acid-binding proteins in rat testes (Ong and Chytil, 1975). This conclusion was reached in part because when retinoic acid is the only form of vitamin A supplied to the rat, the testes undergo the same characteristic changes described above for rats on a total vitamin A-deficient diet. It now appears that retinoic acid has a unique function in the testes. Appling and Chytil (1981) found that retinoic acid is needed in the Leydig cells of the testes for the production of testosterone. They demonstrated that retinol-deficient rats had depressed testosterone levels compared to control rats and retinol-deficient rats supplemented with retinoic acid. In short, retinol is not needed for testosterone production; retinoic acid is.

Ornithine Decarboxylase and the Polyamine Biosynthetic Pathway

Ornithine decarboxylase (EC 4.1.1.17) is the first enzyme, and moreover, the rate-limiting enzyme in the polyamine biosynthetic pathway (Jänne et al., 1978, see Figure 2). It has a molecular weight of 105,000 and is probably a two-subunit protein (Kameji et al., 1982). Of all mammalian enzymes thus far characterized, ODC has the shortest

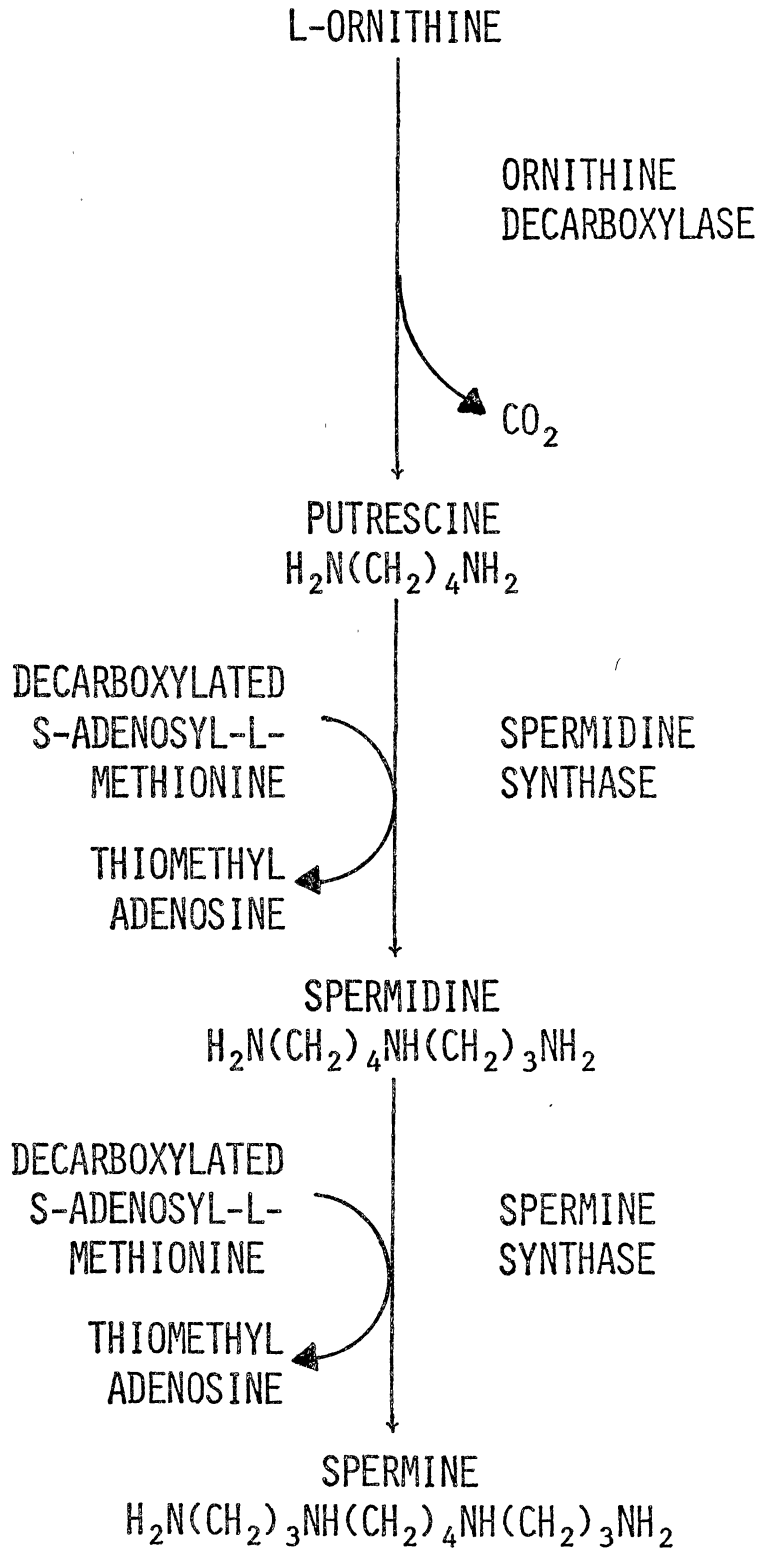


Figure 2. Polyamine Biosynthetic Pathway

half-life, reportedly ranging from 10 to 20 minutes (Russell and Snyder, 1969; Kay and Cooke, 1971). In addition to its extremely short half-life, ODC is hormonally inducible (Reddy and Villee, 1975; Pajunen et al., 1982) and is present in very small amounts in the cell. According to Pritchard et al. (1981), this pyridoxal phosphate-dependent enzyme comprises only 0.000002% of soluble hepatic cellular protein when uninduced. Thus, since it is difficult to obtain ODC in sufficient quantity for study, the turnover is exceptionally rapid, and finally, because ODC is inducible, unravelling the intricate string of events surrounding the role or roles of this enzyme has proven to be a difficult task.

Russell and Snyder (1968) provided evidence associating elevated ODC activity with rapid tissue growth, specifically in regenerating rat liver. After performing partial hepatectomy on rats, the animals were sacrificed and ODC activity determined by measuring the release of $^{14}CO_2$ from labeled ornithine. Those rats sacrificed sixteen hours after partial hepatectomy had ODC activity 25 times greater than control rats.

In addition to elevated ODC activity, increased polyamine levels have been linked to cell proliferation. The three principal polyamines found in mammalian cells are putrescine, spermidine and spermine. As Figure 2 illustrates, the only known metabolic pathway to putrescine is through the decarboxylation of ornithine by ODC. Both spermidine and spermine can then be synthesized from putrescine. Decarboxylated S-adenosyl-L-methionine donates the aminopropyl groups needed by spermidine and spermine synthase for the production of spermidine and spermine, respectively, from putrescine (Pegg et al., 1982).

Mamont et al. (1976) conducted a study that convincingly linked the polyamines to cell proliferation. This group discovered that by competitively inhibiting ornithine decarboxylase with α-methylornithine, they could greatly decrease the concentration of polyamines in hepatoma tissue cells. As a result, the growth of the cells was dramatically retarded. Furthermore, when exogenous polyamines were added to the cells, cell proliferation proceeded normally.

Proposed Mechanisms of Action of Ornithine Decarboxylase

In the last few years several models have been postulated to explain the mechanism of action of ODC. One of the first originated when Dykstra and Herbst (1965) showed that following partial hepatectomy, changes in the concentration of polyamines and RNA closely paralleled each other. Additional light was shed on this finding some years later when Manen and Russell (1975) demonstrated that ODC might control synthesis of nascent RNA through a mechanism involving RNA polymerase I. Recently, these same researchers (Russell and Manen, 1982) theorized that RNA polymerase I, consisting of several subunits, requires a posttranslationally modified ODC to serve as the regulatory subunit s3 of RNA polymerase I. They maintain that ODC is transamidated with four putrescine molecules by transglutaminase on four glutamine residues of the enzyme (Russell, 1981; Russell and Manen, 1982). This complex formed between RNA polymerase I and the ODC-putrescine conjugate and the subsequent increase in ribosomal RNA and protein synthesis is only the end-point, in their opinion, of a trophic cascade of events. Based partly on the fact that ODC is

hormonally induced, Russell et al. (1976) speculate that trophic hormones activate adenylate cyclase, which in turn increases the concentration of cyclic AMP. Cyclic AMP may then activate a protein kinase (Byus and Russell, 1975) that phosphorylates certain proteins associated with the ODC gene and enhances its transcription (Combest and Russell, 1982). More ODC is then available to conjugate with putrescine and thereby stimulate growth as the RNA polymerase I regulatory subunit.

Kuehn and co-workers (Kuehn et al., 1979; Atmar et al., 1981; Atmar and Kuehn, 1981) propose a different model. They maintain that the posttranslational modification of ODC is not a transamidation, but rather a phosphorylation occurring via a polyamine-dependent protein kinase (Daniels et al., 1981). When phosphorylated, Atmar and Kuehn (1981) surmise that ODC is able to form a complex with genes coding for ribosomal RNA which somehow stimulates ribosomal RNA synthesis through action on RNA polymerase I. Kuehn and Atmar (1982) report that spermine and spermidine together activate the protein kinase, which in turn, inactivates ODC. Interestingly, their studies show that putrescine did not activate the kinase, but in fact, countered the activating ability of spermine and spermidine.

Neither of the theories described above is by any means definitive. Other less explored possibilities exist. For example, one group of researchers has discovered what they term ODC antizyme (Fong et al., 1976; Heller et al., 1976). By this model, the polyamines are thought to stimulate the production of the antizyme, which in turn, forms a complex with ODC, thereby rendering the enzyme inactive. On the other hand, the enzyme might be controlled at the level of translation.

Mueckler et al. (1983) described a control mechanism for ornithine aminotransferase, which like ODC, targets ornithine as a substrate. They showed that when aminotransferase is induced, aminotransferase messenger RNA does not increase. Instead, they noted an accelerated rate of protein synthesis on existing messenger RNA. The authors suggest that this could be accomplished in one of two ways: Either a block exists preventing initiation until hormonal induction frees it, or the rate of translation is slowed until induction stimulates more rapid synthesis of the enzyme. Mueckler et al. (1983) provide evidence that in the case of aminotransferase, the latter hypothesis is more likely than the former. Considering the fact that ornithine decarboxylase is also hormonally inducible, one cannot dismiss the possibility that this enzyme is controlled in a similar manner.

Proposed Sites of Action of Vitamin A in ODC Regulation

Numerous mechanisms have been hypothesized to explain the role of vitamin A in supporting cell proliferation and differentiation. For the purposes of this study, only those mechanisms conceivably pertaining to the hypothesized induction modes of ODC and the polyamine biosynthetic pathway will be reviewed. Currently, the most plausible mechanism for vitamin A action is the control of gene expression. A great deal of research has been devoted to determining which gene or genes might be targeted by the retinoids. Even then the critical question is: How does vitamin A exert its effect on a given gene? (Omori and Chytil, 1982).

The retinoids have been implicated in the control of several

enzymes, among them ODC and transglutaminase. Concerning ODC, Russell and Haddox (1981) suggest that retinoid analogs serve to inhibit the transcription of the ODC gene. According to these researchers, when retinoids are added to cells during the first hours of the G_1 phase of the cell cycle, they will inhibit ODC activity, purportedly by inhibiting transcription of the ODC gene for messenger RNA. If added later in G_1 phase, however, ODC activity is not inhibited.

More recent research by the same group (Scott et al., 1982) intimates transglutaminase as a retinoid target. Based on the model described earlier involving the conjugation of putrescine residues to ODC by transglutaminase, Scott et al. (1982) explored the possibility that retinoids were involved in the control of transglutaminase. They found that upon the addition of retinol to Chinese hamster ovary cells, transglutaminase activity increased. More specifically, two transglutaminase peaks were noted in controls. When retinol was added to the ovary cells, the first peak was enhanced. Interestingly, the second peak was actually smaller than those of controls. Furthermore, they reported that a single peak representing ODC activity appeared immediately following the first transglutaminase peak. This second peak was inhibited by 65 percent upon the addition of retinol. Yuspa et al. (1982) have published findings in keeping with those of Scott et al. They established that retinoic acid [Scott et al. (1982) utilized retinol] also resulted in transglutaminase induction in cultured mouse epidermal basal cells. Their purpose, however, was to explore the function of retinoids in regulating epidermal differentiation, since transglutaminase is needed to form the dipeptide bond found in cornified membrane. These researchers

also comment on a possible indirect role for vitamin A in the control of transglutaminase by regulating calcium needed for transglutaminase activity.

Other less explored theories abound. For instance, aside from their postulated role for the retinoids in transglutaminase control, Scott et al. (1982) present an alternate hypothesis for vitamin A action; namely, that cyclic AMP-dependent protein kinases strictly require retinoids in order to move from the cytosol to the nucleus where, by the Russell model, they serve to phosphorylate ODC gene-associated proteins and consequently enhance transcription. Another possibility is offered by Mather (1981) to explain vitamin A action in the testes. He speculates that vitamin A serves to control gonadotropin receptor levels or the manner in which Sertoli and Leydig cells respond to hormones.

Objectives

The objective of this study was to determine if vitamin A exerts an effect on ornithine decarboxylase activity and polyamine concentrations in rat testes. Weanling male rats were placed on a vitamin A-deficient diet until their weight plateaued, at which time one-half were supplemented with retinoic acid and the other half with retinyl palmitate. Those rats supplemented with retinoic acid were expected to resume normal growth, except for the testes, which would continue to degenerate. All tissues, including testicular tissue, of rats supplemented with retinyl palmitate were expected to grow normally. Rats were sacrificed periodically and ODC activity and polyamine concentrations in the testes were measured. Any

differences detected between controls (retinyl palmitate-supplemented rats) and retinoic acid-supplemented rats can be attributed to a retinol deficiency and/or retinoic acid supplementation.

CHAPTER II

METHODS AND PROCEDURES

The objective of this study was to investigate the effect of a retinol-deficient, retinoic acid-supplemented diet on ornithine decarboxylase activity and polyamine levels in testicular tissue of rats. The biochemical methods selected to achieve these ends were considered by the author to provide the most precise and accurate measurements obtainable with the facilities and equipment available at Oklahoma State University.

The overall format for Experiment I was as follows: First, the experimental and control animals were placed on a vitamin A-deficient diet. The rats were weighed periodically to monitor weight gain. When weight gain of the majority of rats reached a plateau for three days, the animals were divided into two groups and supplemented with either retinyl palmitate or retinoic acid. Both groups subsequently resumed normal growth, but since retinoic acid does not support general growth in the testes, these organs continued to degenerate. Animals from both dietary groups were sacrificed at the time of the weight plateau and every few weeks thereafter for 14 weeks (see Figure 3). Following decapitation, the testes were immediately excised from each animal. One testis was used for assay of ODC activity and the other for polyamine analysis. In order to confirm that ODC activity and polyamine concentrations of retinyl palmitate-

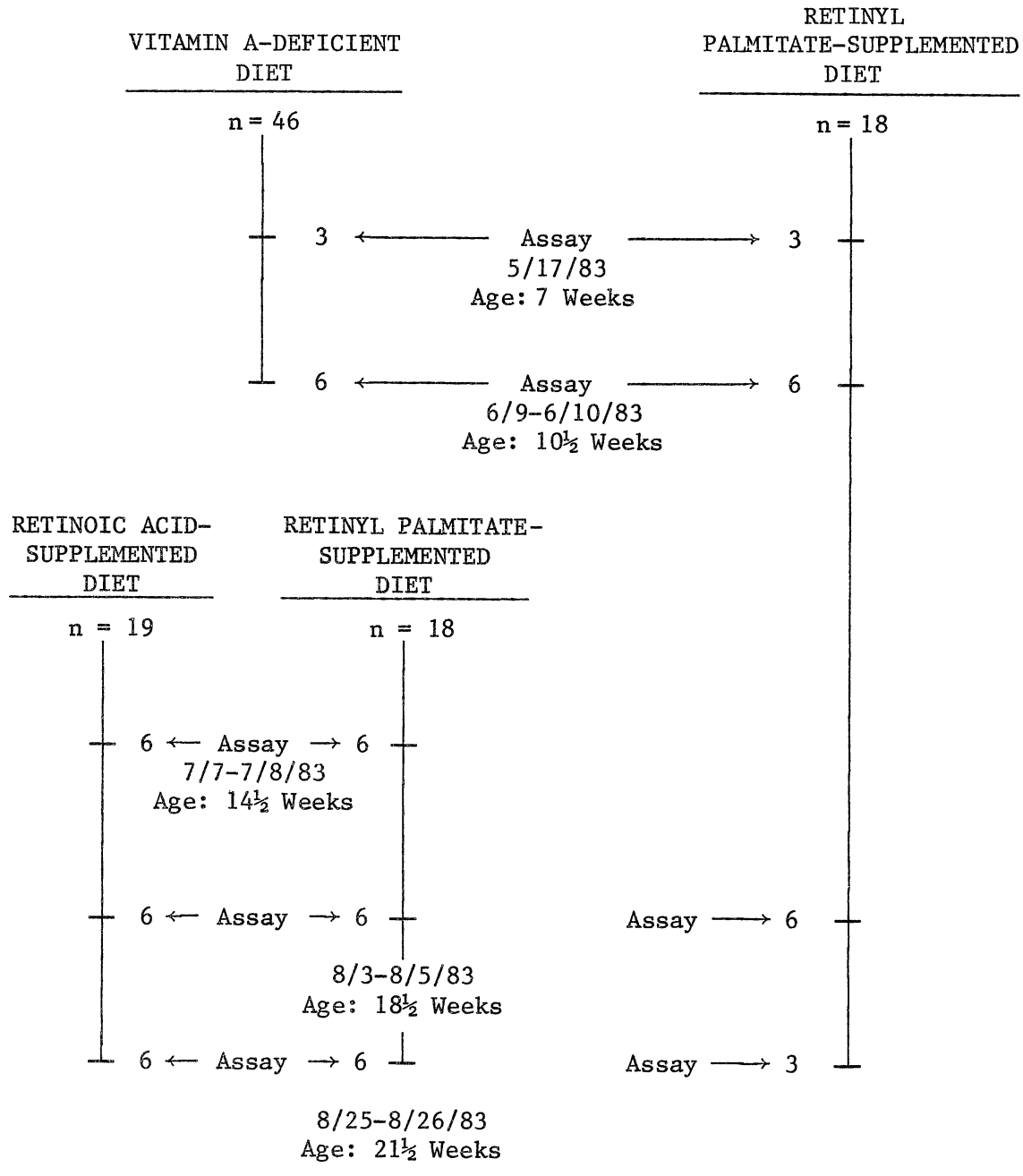


Figure 3. Experimental Plan: Experiment I

supplemented rats were not affected by the weeks spent on a vitamin A-deficient diet, a third group of rats was maintained on a retinyl palmitate-supplemented diet from the time they arrived in the biochemistry department until the end of the study. These rats were sacrificed periodically to assure reliability of the control group.

Animal Care and Diets

Weanling male rats 21 days of age (Sprague-Dawley Holtzman, Charles River Breeding Laboratories Inc., Wilmington, MA) were placed in isolation for one week prior to their transfer to the laboratory animal facility in the biochemistry department. The facility is well ventilated and the temperature was 24-26^{o}C. The lights were on from 6:00 A.M. to 6:00 P.M. and off the alternate 12 hours. Water was available to the animals ad libitum. Metal feeders were placed in each cage. Initially, one feeder was placed in a cage of four animals. However, at the time of the first assay, four weeks after the rats arrived, the animals on the vitamin A-deficient diet weighed significantly more than those maintained on a retinyl palmitate-supplemented diet from the time of their arrival, suggesting that those on the retinyl palmitate diet were not receiving food ad libitum. Hence, from this point on, two feeders were kept in each cage at all times. Furthermore, as the animals grew, they were limited to three, and finally, to two rats per cage. It was necessary, therefore, to purchase another group of rats to be certain that any differences in ODC or polyamine concentration were not attributable to feeding inconsistencies. The same general

format was followed for this experiment (Experiment II) as was followed for Experiment I, except due to space limitations, fewer rats were obtained and assigned to each treatment group (see Figure 4).

The vitamin A-deficient purified diet was prepared for us by TEKLAD (see Appendix A). The retinyl palmitate-supplemented diet was prepared by thoroughly mixing 4.47 mg retinyl palmitate in Rovimix A-325 equivalent to 2.44 mg retinal (Hoffmann-La Roche, Nutley, NJ) per kilogram of the vitamin A-deficient diet. The retinoic acid-supplemented animals received the vitamin A-deficient diet supplemented with 3.09 mg of retinoic acid in stabilized gelatin (Hoffmann-La Roche, Nutley, NJ) per kilogram feed. The animals were weighed weekly until the deficient rats' weight gain slowed, at which time they were weighed every few days until a majority of animals reached a weight plateau. Then approximately one-half of these animals were placed on a retinoic acid-supplemented diet and the other half on the retinyl palmitate-supplemented diet. The animals remained on these diets for the remainder of the study.

Preparation of Animal Tissues

The rats to be sacrificed on a given day were randomly selected from their respective treatment groups. Animals were decapitated between 10:00 A.M. and 11:00 A.M. on each collection day to avoid any diurnal fluctuations in ornithine decarboxylase activity (Hayashi et al., 1972; Noguchi et al., 1979) or polyamine concentrations. Immediately upon decapitation, blood was drained from the neck for serum retinol determination and the testes excised for ODC and

EXPERIMENT II

Arrival Age: 3 Weeks

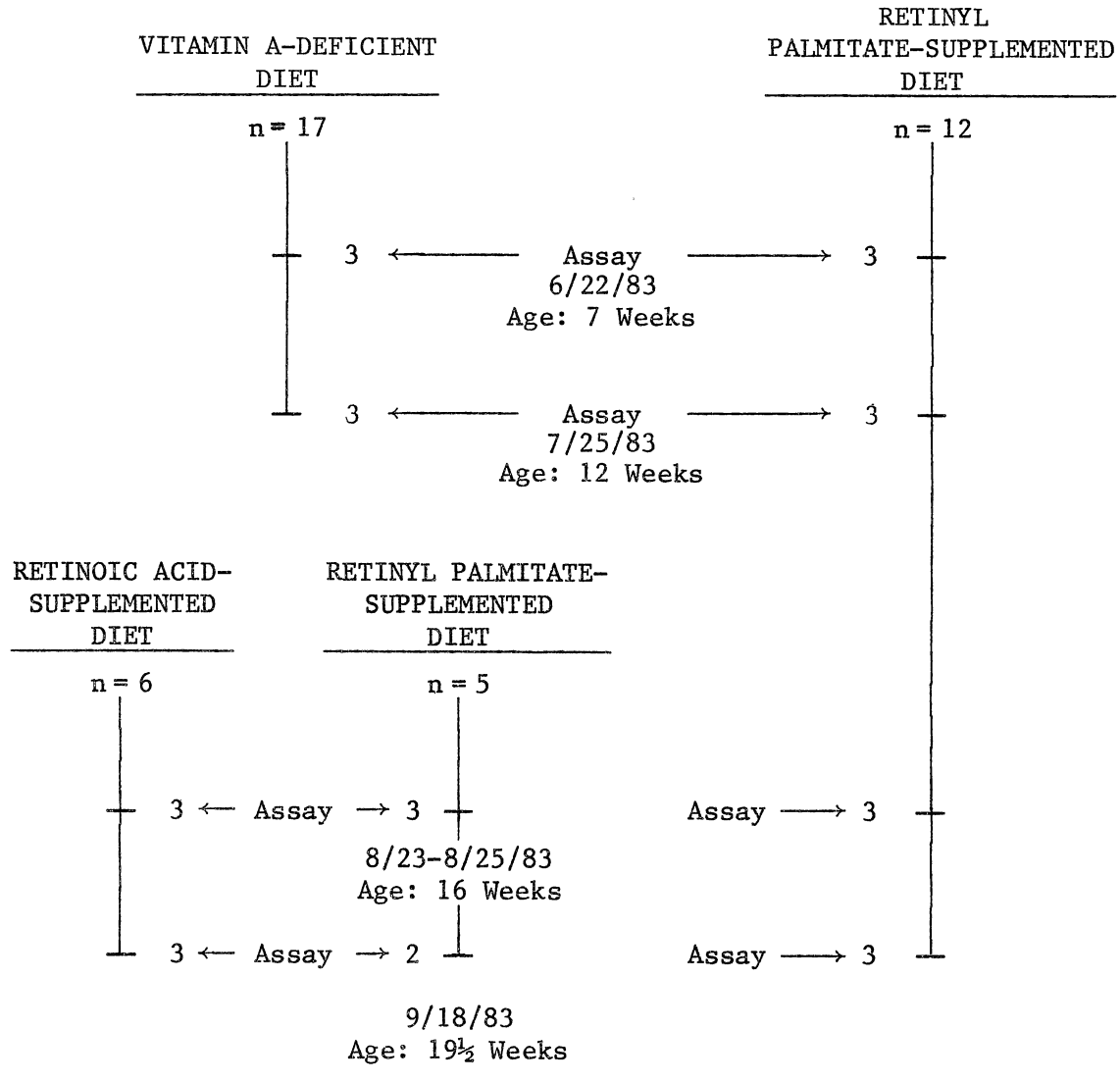


Figure 4. Experimental Plan: Experiment II

polyamine studies. Each testis was weighed. The tunica albuginea was removed on a watch glass over ice. One testis of each rat was placed in four volumes (w/v) of ice cold Tris buffer (25 mM, pH 7.4, containing 0.1 mM disodium EDTA and 1.0 mM dithiothreitol) for ODC activity determination, while the other was placed in four volumes of chilled 10% trichloracetic acid for polyamine analysis. In most cases, the rats per treatment group numbered six for Experiment I and three for Experiment II.

Biochemical Analyses

Specific Activity of Ornithine Decarboxylase

ODC Activity. Ornithine decarboxylase activity was measured essentially by the procedure of Williams-Ashman (Pegg and Williams-Ashman, 1968; Jänne and Williams-Ashman, 1970; Jänne and Williams-Ashman, 1971). The method involves the capture of liberated $^{14}CO_2$ from labeled ornithine. As described above, one testis from each animal was placed in four volumes (w/v) of 25 mM Tris buffer, containing 0.1 mM disodium EDTA and titrated with concentrated hydrochloric acid to a pH of 6.5 at room temperature, which at the later incubation temperature of 37°C was 7.4. Just before conducting the experiment, 1.0 mM dithiothreitol (Aldrich Chemical Co., Milwaukee, WI) was added to the buffer to stabilize the enzyme.

Each testis was homogenized with ten passes of a teflon pestle in a glass Potter-Elvehjem homogenizer which was kept in ice throughout the process. The homogenate was then transferred to tubes and centrifuged for 30 minutes at 30,000 x g at 4°C. The

resulting supernatant solution containing the enzyme was poured into vials and kept on ice briefly until added to the incubation mixture. The incubation mixture, except for the enzyme, was prepared prior to the start of the experiment in the following manner: First, since ODC is a pyridoxal phosphate-requiring enzyme, 50 µl of a 0.002 M (0.1 µmol) solution of pyridoxal-5-phosphate (Sigma Chemical Co., St. Louis, MO) were delivered to the bottom of test tubes fitted with disposable rubber stoppers and polypropylene center wells (Kontes Glass Co., Vineland, NJ). Second, 50 µl of a 0.05 M solution (2.5 µmol) of dithiothreitol were added to stabilize the enzyme. Third, 150 µl of Tris buffer were added. Fourth, a mixture of DL-[1-^{14}C]ornithine hydrochloride (specific activity 56 to 58 mCi/mmol, Amersham Corp., Arlington Heights, IL) and L-ornithine (Sigma Chemical Co., St. Louis, MO) was prepared to yield 0.5 µmol L-ornithine and 0.3 µCi per 50 µl aliquot. This was also delivered to the bottom of the test tubes, bringing the volume of the incubation mixture to 0.3 ml. While the homogenate was centrifuging, the polypropylene center wells were filled with 0.2 ml of hyamine hydroxide (Aldrich Chemical Co., Milwaukee, WI). The test tubes were then lightly stoppered pending the addition of the enzyme to the incubation mixture. At this point, 0.2 ml of the supernatant fluid was added to duplicate tubes containing the incubation mixture to bring the final volume to 0.5 ml. Two non-enzymatic controls were prepared by adding 0.2 ml Tris buffer in place of the enzyme. The tubes were immediately stoppered and placed in a 37^{o}C shaking waterbath for 60 min. A stopwatch was used to mark the time. After one hour, the reaction was terminated by injecting 0.5 ml of 40%

trichloracetic acid by needle through the rubber stopper. The test tubes were permitted to stand several hours to allow time for trapping of all CO_2. Next, 10 ml of Insta-gel (Packard Instrument Co., Downers Grove, IL) were placed in 20 ml scintillation vials. The hyamine hydroxide-containing center wells were clipped into the scintillation fluid and the vials tightly capped. The vials were placed in the dark and left for two to three days to allow time for equilibration before being counted on a Packard Counter, Model 2425 (Packard Instrument Co., Downers Grove, IL). An ESCR quench correction curve was prepared with standards, and used to determine efficiency and from that, disintegrations per minute (dpm) of the unknown samples. Since the specific activity of ODC in picomoles CO_2 per mg protein per hour was desired, the protein concentration of the enzyme-containing supernatant fluid was also determined.

Protein Determination. The Bradford procedure (Bradford, 1976), also referred to as the Coomassie Blue method, was utilized to determine the protein concentration of the ODC supernatant solution obtained in the above procedure. The advantages of this procedure are that it is rapid and chemical interference is slight. The disadvantage is that the protein/protein variation is significant (Bio Rad Technical Bulletin, 1979).

The supernatant fluid was frozen following the removal of the 0.2 ml aliquots needed for ODC activity determination in the above procedure. Immediately prior to protein determination, the supernatant solution was removed from the freezer and permitted to thaw

at room temperature.

The Coomassie Blue reagent was used within two weeks after preparation. Fifty milligrams of Coomassie Brilliant Blue G-250 (Sigma Chemical Co., St. Louis, MO) were dissolved in 25 ml of 95% ethanol. Next, 50 ml of phosphoric acid (85% w/v) were slowly added and thoroughly mixed. The solution was brought to a final volume of 50 ml with distilled water. Standards were made from bovine serum albumin (10 g/dl in 0.85% sodium chloride solution) obtained from Sigma Chemical Co., St. Louis, MO. A portion of the stock standard (10 g/dl) was diluted 100 fold in a volumetric flask with 0.85 g/dl sodium chloride solution, resulting in a final concentration of 100 µg/0.1 ml. Further dilutions were necessary to obtain the desired concentrations:

µg protein/0.1 ml	Standard (100 µg/0.1 ml) (ml)	0.85 g/dl NaCl (ml)
100	10	0
80	8	2
60	6	4
40	4	6
20	2	8

In each case, the designated amount of standard was pipetted into a 10 ml volumetric flask and filled to volume with 0.85 g/dl NaCl. After mixing reagent and standards, it was necessary to determine what dilution of the enzyme-containing supernatant solution from the ODC procedure was required to make the samples fall on the upper one-half of the standard curve. This was determined to be a 1/15 dilution. Therefore, each sample was diluted 1/15 with 0.85 g/dl sodium chloride solution, before conducting the assay. Standards and samples were assayed in duplicate. Then, 0.1 ml was pipetted into disposable 13

x 100 mm disposable culture tubes (Lancer, St. Louis, MO). To this 5 ml of Coomassie Blue reagent, which was filtered just before beginning the procedure to remove any precipitate, were added to each tube and vortexed immediately. A blank was prepared by pipetting 0.1 ml of 0.85 g/dl sodium chloride solution into tubes and proceding as for standards and samples. After two minutes and within one hour after adding Coomassie Blue, the absorbance of the samples and standards was read at a wavelength of 595 nm on a spectrophotometer. A standard curve was drawn by plotting known µg protein of the standards against the corresponding absorbance. The protein concentration of the unknown samples was subsequently determined from this curve.

Calculation of Specific Activity of ODC. The disintegrations per minute (dpm) were calculated by subtracting the control counts per minute (cpm) from the sample cpm and dividing by the efficiency determined from the ESCR quench correction curve. The dpm value used to calculate the specific activity of each sample was the average dpm of the duplicates:

$$\text{ODC Specific Activity } \frac{\text{pmol } CO_2}{\text{mg protein/hr}} = \frac{\text{dpm}}{0.2 \text{ ml/hr}} \times \frac{\text{mCi}}{2.22 \times 10^9 \text{ dpm}}$$

$$\times \frac{\text{mmol ornithine}}{\text{mCi}} \times \frac{\text{mol}}{10^3 \text{ mmol}} \times \frac{10^{12} \text{ pmol}}{\text{mol}} \times \frac{\text{ml}}{\text{mg protein}}$$

Polyamine Analysis

Polyamine Quantitation on Amino Acid Analyzer. Polyamines were quantitated on an amino acid analyzer equipped with an integrator. The method used was similar to that described by Dunzendorfer and

Russell (1978). However, these researchers suggest a four-buffer system. In addition, Dunzendorfer and Russell utilized a fluorescence detector assembly, requiring a fluorescence reagent, in this case o-phthalaldehyde. This study did not have access to a fluorescence detector. Thus, ninhydrin was used instead of o-phthalaldehyde. Furthermore, for the purposes of this study, a two-buffer system was adequate.

As described previously, at the time of sacrifice, one testis of each rat was placed in four volumes of 10% trichloracetic acid. The testis was homogenized in this solution with twelve passes in a Potter-Elvehjem homogenizer with a teflon pestle. The homogenates were placed in centrifuge tubes and centrifuged at 30,000 x g for thirty minutes. The supernatant fluid was poured into vials and placed in the freezer until needed for polyamine quantitation. The pellet was left in the centrifuge tube and frozen for later DNA determination.

The stock buffer (Buffer 2) was a 2.4 M potassium chloride, 0.09 M potassium citrate solution, containing five ml thiodiglycol (Pierce Chemical Co., Rockford, IL) per liter and titrated to pH 5.6 with concentrated hydrochloric acid. The other buffer of the two-buffer system (Buffer 1) was prepared by diluting the stock buffer by half to a 1.2 M potassium chloride, 0.045 M potassium citrate solution.

Putrescine, spermidine and spermine (Sigma Chemical Co., St. Louis, MO) served as standards. Each was dissolved in 0.1 N hydrochloric acid to a concentration of 2.5 mM. One milliliter of each standard was placed in the same 10 ml volumetric flask and filled to volume with 0.1 N hydrochloric acid for a final concentration of 0.25

mM or 250 nm/ml of each standard.

The ninhydrin reagent was prepared by mixing 75 ml sodium acetate (4 N, pH 5.51), 225 ml DMSO (Pierce Chemical Co., Rockford, IL), 150 ml water and 6.05 g ninhydrin (Pierce Chemical Co., Rockford, IL). At this point, the solution was connected to the amino acid analyzer, 0.3 g hydrindantin (Pierce Chemical Co., Rockford, IL) added, and the bottle immediately clamped to prevent oxidation. Finally, the mixture was bubbled with N_2 for several hours with stirring.

Cation exchange resin PA-35 was purchased from Beckman (Palo Alto, CA). The column height was 7 cm and the diameter, 2.6 mm. During analysis, the temperature of the column was maintained at 65^{o}. The polyamines were detected at two wavelengths, 440 nm and 570 nm.

To equilibrate the column, Buffer 1 was run for 20 to 30 minutes at a flow rate of 0.1 ml/minute. This flow rate was kept constant throughout the procedure. Following equilibration, 100 µl of standard was injected by automatic injector and Buffer 1 was run 33 minutes to elute putrescine. Then Buffer 2 was used for 30 minutes to elute spermidine and spermine, respectively. Buffer 1 was used between runs to re-equilibrate the column. When ready to assay the samples, the vials were removed from the freezer and permitted to thaw at room temperature. The thawed samples were thoroughly vortexed and approximately 1.5 ml were transferred to teflon microfuge tubes and microfuged about one minute to remove any precipitate. Samples were injected and analyzed in precisely the same way as standards. The peak area analysis was carried out by integrator, based on absorbance at both 440 and 570 nm, and

quantities reported in nmoles/ml. As little as 0.2 nmol can be detected by this method.

DNA Determination. Preliminary tissue preparation was achieved by a method similar to the Schmidt-Thannhauser procedure (Schmidt and Thannhauser, 1945), modified by Fleck and Munro (Fleck and Munro, 1961; Munro and Fleck, 1966). The pellet remaining after centrifugation of the homogenate described in the polyamine procedure was removed from the freezer and thawed at room temperature. It was washed twice with 4 ml of 10% trichloracetic acid and centrifuged after each wash at 1420 x g for 30 minutes. A solution of 0.3 N potassium hydroxide was made with potassium hydroxide crystals. Ten milliliters were added to each pellet and the pellet dissolved completely. Five milliliters of this solution were placed in a 10 ml volumetric flask and 2.4 ml of 0.3 N potassium hydroxide solution added. The flask was filled to volume with distilled water. Equal volumes (3 ml) of this solution and 1.2 N perchloric acid (prepared from 70-72% perchloric acid) were poured into 20 ml vials and placed in a 70^{o}C waterbath for 20 minutes.

Highly polymerized calf-thymus DNA (Sigma Chemical Co., St. Louis, MO) was used for standards. Twenty milligrams of this DNA were dissolved in 50 ml 5.0 mM sodium hydroxide, resulting in a 0.4 mg/ml concentration. This stock standard was used to prepare working standards:

DNA Concentration (µg/ml)	Stock Standard (ml)	Water (ml)	0.6 N Perchloric Acid (ml)
0	0	10.00	10.00
10	0.50	9.50	10.00
20	1.00	9.00	10.00
50	2.50	7.50	10.00
70	3.50	6.50	10.00
100	5.00	5.00	10.00
120	6.00	4.00	10.00
150	7.50	2.50	10.00

The standards were heated at 70°C for 20 minutes, stored at 4°C, and used within 3 weeks.

After preparing the standards and samples as described above, the diphenylamine reaction as outlined by Burton (1955) was employed to estimate DNA content per testis colorimetrically. The diphenylamine reagent is comprised of 1.5 g diphenylamine (Sigma Chemical Co., St. Louis, MO), 100 ml glacial acetic acid and 1.5 ml concentrated sulfuric acid. Just before using, 0.1 ml aqueous acetaldehyde (two ml acetaldehyde diluted to 100 ml with distilled water) per 20 ml reagent was added. One milliliter each of samples and standards was pipetted into disposable test tubes, in duplicate. Two milliliters of the above reagent were then added to each tube and the mixture thoroughly vortexed. The reaction was permitted to proceed for 13 to 15 hours before measuring absorbance at 600 nm. The known µg/ml of the standards was plotted against absorbance values. The resulting curve was used to determine the concentration of the unknown samples.

Serum Retinol Determination

Serum retinol was measured by the method of Bieri et al. (1979).

Statistical Methods

Means in different experimental groups were compared using Student's t test (two-tailed) (Snedecor and Cochran, 1967). Unless otherwise noted, alpha equals .05 for all statistical analyses.

CHAPTER III

RESULTS

In an effort to understand the role of vitamin A in general growth and reproduction, researchers have strived to elucidate the mode of action of this vitamin. Despite the efforts of numerous investigators, the manner in which vitamin A exerts its effect remains an enigma. However, some noteworthy associations have been made; among them the fact that vitamin A, elevated polyamine levels and increased ornithine decarboxylase activity have all been linked to growth (Wohlbach and Howe, 1925; Russell and Snyder, 1968; Jänne and Raina, 1968; Jänne et al., 1978). With this in mind, the goal of this study was to examine the effect of retinol deficiency and/or retinoic acid supplementation on ODC activity and polyamine levels in the testicular tissue of rats. Significant differences in these parameters between control and experimental animals would provide evidence that these factors are related, and further, pave the way for additional studies to determine the mechanism of action of vitamin A, ODC and the polyamines.

Animal Weight

As described in detail in the previous chapter, the overall experimental plan was to bring rats to vitamin A deficiency by omitting the vitamin from the diet. Animals were considered deficient, or near

deficiency, when their weight reached a plateau for several days. At this point, the primary control group began receiving its usual feed supplemented with retinyl palmitate. In contrast, the experimental animals received retinoic acid in the diet. As a check, to be certain that the initial state of deficiency of control animals had no effect on results, a third group of rats received the vitamin A-deficient diet supplemented with retinyl palmitate throughout the study. Figure 5 illustrates the weight of the animals to be used on each scheduled day of assay. As can be noted, the first assay was conducted when the rats were seven weeks old. These animals had been consuming either the retinyl palmitate-supplemented diet or vitamin A-deficient diet for four weeks. This assay was conducted to monitor the progress of the animals and to establish that there were no significant differences in weight, ODC activity or polyamine concentration. Surprisingly, this was not the case. The rats consuming the retinyl palmitate-supplemented diet weighed less [169 g ± 12.5 (SD)] than those on the vitamin A-deficient diet (246 g ± 26.6). Furthermore, the specific activity of ODC was significantly greater in those animals on the vitamin A-deficient diet [603 pmol CO_2/mg protein/hr ± 113 (SD)] than those consuming the retinyl palmitate-supplemented diet (208 pmol CO_2/mg protein/hr ± 143). The most probable explanation for this discrepancy is inadequate or inconsistent feeding of the animals receiving the retinyl palmitate-supplemented diet. The problem was rectified as described earlier; additional metal feeders were placed in each cage and more rats purchased to conduct Experiment II. Figure 6 shows the weight of rats on scheduled days of assay for Experiment II. In contrast to Experiment I, the weights

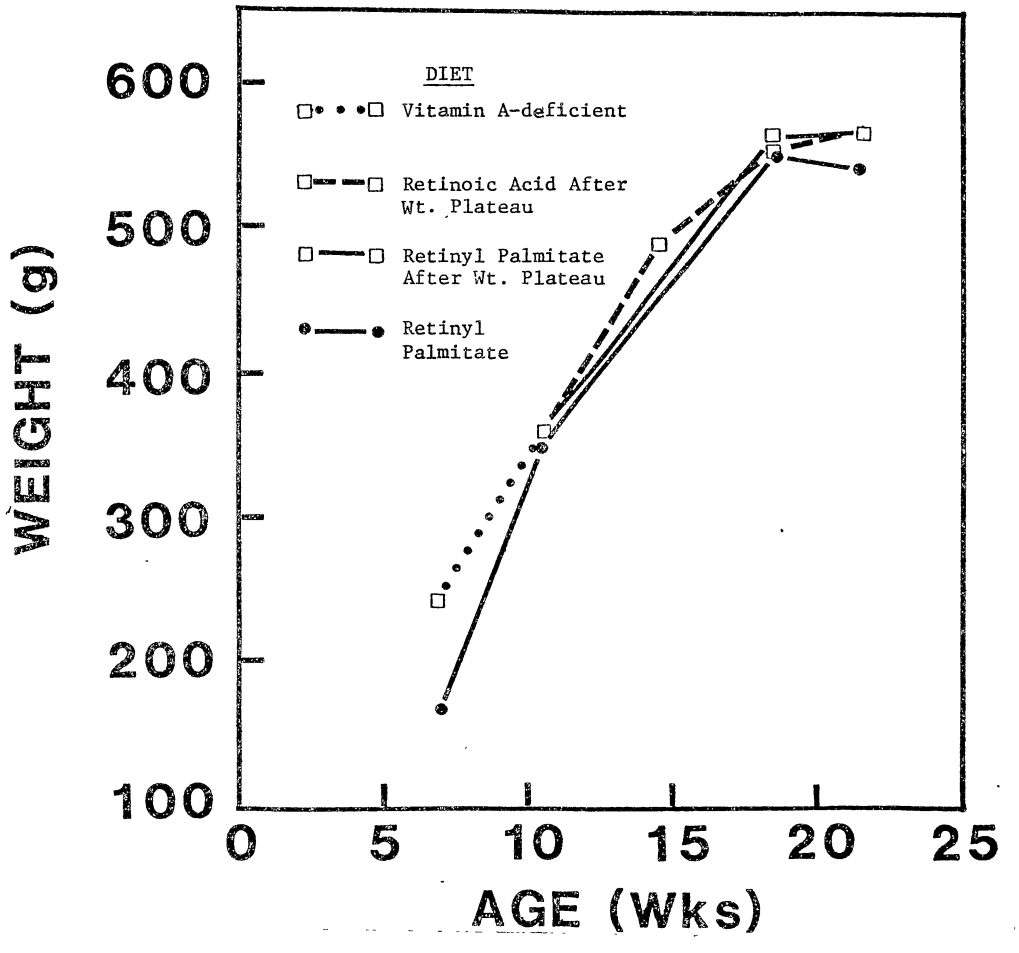


Figure 5. Mean Weight of Rats at Time of Assay: Experiment I

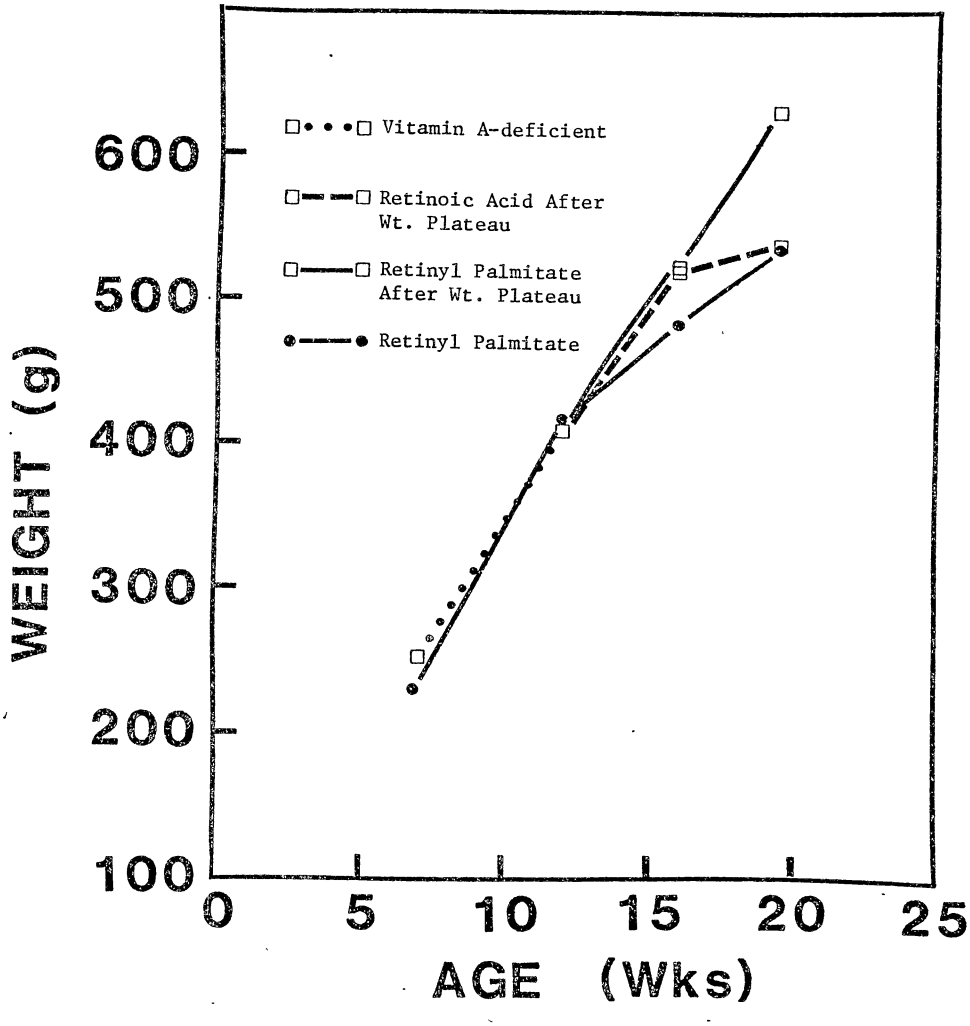


Figure 6. Mean Weight of Rats at Time of Assay: Experiment II

were not different at seven weeks of age.

After the feeding problem was discovered and rectified, there is evidence to indicate that it had no long-term deleterious effects. First of all, the weights of control animals that were receiving retinyl palmitate throughout Experiment I caught up to those animals on the deficient diet by the time the second sacrifice was conducted. In addition, ODC activity of the two treatment groups was not different at this time.

Although it is difficult to be absolutely certain that the animals on the vitamin A-deficient diet at the beginning of Experiment I were not also deprived of adequate feed at some time, if one compares the weights of the rats consuming this diet in Experiments I and II at seven weeks of age, the point at which the error was discovered and remedied, the mean weight of the rats in Experiment I was 246 g ± 26.6 (SD), while that of the animals in Experiment II was 256 g ± 7.21. The difference is not significant. In contrast, those animals in Experiment I consuming the retinyl palmitate-supplemented diet weighed 169 g ± 12.5 at seven weeks of age, compared to 232 g ± 12.1 in Experiment II. In this instance, a significant difference is noted. In short, based on weight gain and ODC specific activity, it seems reasonable to conclude that the overall effect of limited feeding on the results of Experiment I was minimal or non-existent.

Serum Retinol

Serum retinol determinations were conducted for two reasons. First, it was essential to verify that those animals presumed to be retinol-deficient throughout the study had not received retinol in

their diet by mistake. Such an error could have disastrous effects on the outcome of the study. Second, it was important to confirm that at the weight plateau, the rats on the vitamin A-deficient diet were near retinol deficiency, and moreover, that they continued to complete deficiency after being placed on a retinoic acid-supplemented diet, or returned to normal serum retinol levels if supplemented with retinyl palmitate. Tables I and II show individual retinol values and means for Experiments I and II, respectively. As can be seen, at the weight plateau, the rats consuming the vitamin A-deficient diet in both experiments were deficient. The mean serum retinol concentration for these rats in Experiment I at the weight plateau was 3.7 µg/dl; for Experiment II, 6.5 µg/dl. In contrast, those rats receiving retinyl palmitate in their diet from the start of the study had serum retinol levels of 65 µg/dl for Experiment I and 63 µg/dl for Experiment II. Furthermore, after the animals consuming the deficient diet were placed on either a retinyl palmitate-supplemented or retinoic acid-supplemented diet, none of those receiving retinoic acid had any measurable serum retinol at the time of assay throughout the remainder of the study, while those consuming retinyl palmitate had normal vitamin A levels at the time of the assay.

The author acknowledges that the technique employed to obtain serum for analysis (draining blood from the neck following decapitation) would not be satisfactory if a highly accurate determination were necessary. It is likely that other tissue fluids besides vascular contents contaminated the sample. But, it was critical that the animal not be traumatized since ornithine decarboxylase is hor-

TABLE I

SERUM RETINOL CONCENTRATION AND MEAN FOR RATS IN EXPERIMENT I

Age	Diet								
	Vitamin A-Deficient			Retinyl Palmitate-Supplemented					
	n	Serum Retinol (µg/dl)	Mean (µg/dl)				n	Serum Retinol (µg/dl)	Mean (µg/dl)
7 Weeks	3	38, 13, 42	31				3	49, 56, 49	51
10½ Weeks	6	4.4, 5.6, 6.0 -[2] -[2] 6.1	3.7				6	53, 79, 68 75, 64, 53	65
	Diet[1]								
	Retinyl Palmitate-Supplemented			Retinoic Acid-Supplemented					
	n	Serum Retinol (µg/dl)	Mean (µg/dl)	n	Serum Retinol (µg/dl)	Mean (µg/dl)			
14½ Weeks	6	34, 45, 62 38, 72, 42	49	6	-[2]	-[2]			
18½ Weeks	6	72, 58, 62 69, 54, 54	62	6	-[2]	-[2]	6	47, 75, 56 53, 55, 42	63
21½ Weeks	6	51, 62, 39 59, 43, 32	48	6	-[2]	-[2]	3	30, 56, 43	43

[1] Vitamin A-deficient rats were placed either on retinyl palmitate- or retinoic acid-supplemented diets at 10½ weeks when they reached weight plateau.

[2] Non-measurable.

TABLE II

SERUM RETINOL CONCENTRATION AND MEAN FOR RATS IN EXPERIMENT II

	Diet					
	Vitamin A-Deficient			Retinyl Palmitate-Supplemented		
	n	Serum Retinol (μg/dl)	Mean (μg/dl)	n	Serum Retinol (μg/dl)	Mean (μg/dl)
7 Weeks	3	30, 49, 58	46	3	66, 83, 47	65
12 Weeks	3	5.2, 9.0, 5.2	6.5	3	74, 68, 46	63

	Diet[1]								
	Retinyl Palmitate-Supplemented			Retinoic Acid-Supplemented					
	n	Serum Retinol (μg/dl)	Mean (μg/dl)	n	Serum Retinol (μg/dl)	Mean (μg/dl)	n	Serum Retinol (μg/dl)	Mean (μg/dl)
16 Weeks	3	60, 68, 82	70	3	-[2]	-[2]	3	63, 58, 60	60
19½ Weeks	2	57, 41	49	3	-[2]	-[2]	3	69, 70, 55	65

[1] Vitamin A-deficient rats were placed either on retinyl palmitate- or retinoic acid-supplemented diets at 12 weeks when they reached weight plateau.

[2] Non-measurable.

monally inducible. Hence, the blood could not be removed before decapitation. In fact, decapitation was selected as the means of sacrifice rather than cervical dislocation or other methods for the same reason; the less trauma to which the animal was subjected, the less chance that ornithine decarboxylase activity would be affected by hormonal induction or other stimuli. In short then, for the purposes of this study, the technique was adequate and provided a means to monitor retinol levels in the blood.

Ornithine Decarboxylase Activity

A number of studies have confirmed that ornithine decarboxylase activity in various tissues fluctuates in animals of the same age (Hayashi et al., 1972; Tabor and Tabor, 1976; Osterman et al., 1983). However, Osterman et al. (1983) reported that the enzyme activity in testes of the same rat varied only 2.2%. Therefore, this study utilized one testis for ornithine decarboxylase determination and one testis for polyamine analysis. The mean specific activity of the enzyme in Experiments I and II is reported in Tables III and IV, respectively. The results show that on no day of sacrifice in either experiment were the mean ODC specific activities different for what might be termed the control and the "control for the control"; in other words, the group of rats receiving retinyl palmitate in their diet from the time of the deficient rats' weight plateau and the animals consuming retinyl palmitate in their diet throughout the study, respectively. This is pertinent for two reasons. In the first place, these results indicate that the primary control group was not affected, in terms of weight and ODC activity, by the weeks

TABLE III

MEAN ODC SPECIFIC ACTIVITY ± SD OF RAT TESTES FOR EXPERIMENT I

Age	Diet					
	Vitamin A-Deficient				Retinyl Palmitate-Supplemented	
	n	Mean ODC Specific Activity ($\frac{\text{pmol } CO_2}{\text{mg protein·hr}}$) ± SD			n	Mean ODC Specific Activity ($\frac{\text{pmol } CO_2}{\text{mg protein·hr}}$) ± SD
7 Weeks	3	603 ± 113*			3	208 ± 143
10½ Weeks	6	960 ± 185			6	947 ± 88.6
	Diet[1]					
	Retinyl Palmitate-Supplemented		Retinoic Acid-Supplemented			
	n	Mean ODC Specific Activity ($\frac{\text{pmol } CO_2}{\text{mg protein·hr}}$) ± SD	n	Mean ODC Specific Activity ($\frac{\text{pmol } CO_2}{\text{mg protein·hr}}$) ± SD		
14½ Weeks	6	506 ± 229	6	$1.30 \times 10^3 \pm 833$**		
18½ Weeks	6	584 ± 143	6	$3.15 \times 10^3 \pm 1.05 \times 10^3$*	6	728 ± 279
21½ Weeks	6	410 ± 145	6	992 ± 369*	3	413 ± 94.0

*Significantly different from controls at $\alpha = .05$.

**Significantly different from controls at $\alpha = .10$.

[1]Vitamin A-deficient rats were placed either on retinyl palmitate- or retinoic acid-supplemented diets at 10½ weeks when they reached weight plateau.

TABLE IV

MEAN ODC SPECIFIC ACTIVITY ± SD OF RAT TESTES FOR EXPERIMENT II

Age	Vitamin A-Deficient		Retinyl Palmitate-Supplemented	
	n	Mean ODC Specific Activity ($\frac{\text{pmol } CO_2}{\text{mg protein} \cdot \text{hr}}$) ± SD	n	Mean ODC Specific Activity ($\frac{\text{p mol } CO_2}{\text{mg protein} \cdot \text{hr}}$) ± SD
7 Weeks	3	607 ± 129	3	540 ± 58.9
12 Weeks	3	523 ± 156	3	556 ± 122

	Diet[1]					
	Retinyl Palmitate-Supplemented		Retinoic Acid-Supplemented			
	n	Mean ODC Specific Activity ($\frac{\text{pmol } CO_2}{\text{mg protein} \cdot \text{hr}}$) ± SD	n	Mean ODC Specific Activity ($\frac{\text{pmol } CO_2}{\text{mg protein} \cdot \text{hr}}$) ± SD		
16 Weeks	3	389 ± 170	3	421 ± 102	3	391 ± 124
19½ Weeks	2	560 ± 276	3	883 ± 610	3	387 ± 177

[1]Vitamin A-deficient rats were placed either on retinyl palmitate- or retinoic acid-supplemented diets at 12 weeks when they reached weight plateau.

spent on the deficient diet prior to being supplemented with retinyl palmitate in either experiment. To secure this assurance was the sole purpose for including a treatment group sustained on retinyl palmitate-supplemented feed throughout the study. Secondly, it provides further evidence of the validity of Experiment I in spite of the feeding mishap, in that ODC specific activity is the same, as would be expected, for the misfed control group (retinyl palmitate-supplemented throughout the study) and the primary group supplemented with retinyl palmitate after nearing vitamin A deficiency.

One other inconsistency must be dealt with at this point. At the weight plateau, one-half the rats on the deficient diet were supplemented with retinyl palmitate, while the other one-half received the retinoic acid-supplemented diet. Those receiving retinoic acid began to exhibit the characteristic symptoms of retinol deficiency in the testes soon after supplementation. Specifically, degeneration gradually ensued along with signs of edema, which eventually became severe. At the time of the first assay following supplementation of the vitamin A-deficient animals, the testes of retinoic acid-supplemented rats exhibited no noticeable edema. But, by the second assay following supplementation edema was marked, although the extent of the condition varied considerably from rat to rat. Unfortunately, this researcher failed to include the edematous fluid during this second assay following supplementation in Experiment I. In retrospect, it is obvious that this fluid should be included in order for the calculated specific activity to reflect the activity of the entire testis. Not only were certain components of the fluid possibly lost, but the protein concentration of the ODC supernatant solution obtained in the

ODC procedure is used to calculate specific activity of the enzyme; thus, this oversight could adversely affect the results. Upon realizing the possible consequences of this action, a final assay was conducted and the edematous fluid carefully included in the testis-buffer homogenate obtained during the ODC procedure. It should be noted that removing the tunica albuginea from the testis without losing any of the edematous fluid was extremely difficult. One cannot dismiss the possibility that minute amounts were lost during the procedure. The only treatment group on which this had any bearing was the group receiving the retinoic acid-supplemented diet. Furthermore, in all instances in Experiment II, the testicular edematous fluid of rats supplemented with retinoic acid was included in the assay.

As previously discussed, the ODC activity of the control groups was as expected; no differences. This was not the case for the remainder of the ODC activity data. To begin with, as discussed in detail previously, the mean weight of the rats consuming the retinyl palmitate-supplemented diet was significantly less than the weight of those receiving the deficient diet at the time of the first assay (at seven weeks of age) of Experiment I. Under these circumstances, it was not surprising that the ODC activity in the testes of the retinyl palmitate-supplemented animals was significantly lower (208 pmol CO_2/mg protein/hr) than that of those on the deficient diet (603 pmol CO_2/mg protein/hr). Activity of ODC has been associated with proliferating tissue (Russell and Snyder, 1968), and during the first few weeks of the study, the supplemented rats were not receiving adequate feed for normal growth. By the time of the second assay,

weights of the animals on the two diets were no longer significantly different, nor were levels of ODC activity (960 pmol CO_2/mg protein/hr ± 185 for the vitamin A-deficient animals; 947 pmol CO_2/mg protein/hr ± 88.6) for the retinyl palmitate supplemented rats. Findings of Experiment II up to and including data obtained at the time the deficient rats reached weight plateau were more consistent with expected results than corresponding findings of Experiment I. That is, at seven weeks of age and at the weight plateau, neither weight nor specific activity was significantly different for animals on the vitamin A-deficient and retinyl palmitate-supplemented diets. This is what had been expected in Experiment I to confirm that the initial few weeks of deficiency for both control and experimental animals would have no adverse effects on subsequent findings after supplementation with retinyl palmitate or retinoic acid was instigated.

The most striking data were those obtained once supplementation began. Assays were conducted for Experiment I when the rats were 14½, 18½ and 21½ weeks of age (See Table III). At 14½ weeks, the rats previously receiving the vitamin A-deficient diet had been receiving either the retinyl palmitate or retinoic acid supplementation for four weeks. At this time, there was not a significant difference in ODC activity, but a distinct tendency for greater ODC activity in the testes of retinoic acid-supplemented animals was observed ($P < .10$). At this point, very little, if any, edema was noted. When the rats were 18½ weeks old, the assay was conducted in which the edematous fluid of the degenerating testis of the retinoic acid-supplemented rat was not included. In this instance, the testicular ODC specific activity was significantly greater in the experimental group than in

the controls. A final assay conducted when the animals reached 21½ weeks of age included the edematous fluid contained in the testis. Again, the enzyme activity in the retinoic acid-supplemented rats was significantly greater than in the controls. These results are extremely interesting, considering the fact that increased ODC activity is associated with cell proliferation (Russell and Snyder, 1968), while in this instance the degenerating organ exhibits greater activity than the control undergoing normal growth.

The data obtained in Experiment II are less definitive. Due to the limitations outlined earlier, fewer rats were allotted to each treatment group. Moreover, only three animals from each treatment group (except for one instance when there were only two) were assayed. Finally, where in Experiment I, three rats were assayed following supplementation of the deficient animals, in Experiment II there were only enough animals to schedule two days for assay after adding retinoic acid or retinyl palmitate to the diets of the deficient rats. The first assay was conducted four weeks after supplementation was begun, as in Experiment I. No difference between ODC activity of control and experimental groups was observed. The final assay for Experiment II occurred when the animals were 19½ weeks old. No significant difference in ODC activity was observed with the statistical methods utilized, although an apparent trend ($P < .15$) is in agreement with the findings of Experiment I; namely, that the retinoic acid-supplemented rats exhibit greater ODC specific activity in the testes than do rats supplemented with retinyl palmitate.

Polyamine Analysis

A representation of a typical chromatogram of standards and samples is illustrated in Figures 7 and 8. Twenty-five nanomoles of each standard were injected in a volume of 100 µl onto the amino acid analyzer. The integrator, connected to the analyzer, was programmed to record concentration in nmol per ml, as well as elution time and KF, which are indicated on the figures. The average elution times for the three standards are tabulated below.

TABLE V

MEAN (MIN) ELUTION TIME FOR POLYAMINE STANDARDS

Polyamine	Mean
Putrescine	30.4
Spermidine	42.3
Spermine	52.1

Standards were run each day samples were analyzed, since peak areas varied slightly based on freshness of ninhydrin. Polyamine concentrations were calculated per mg DNA. The results are recorded in Tables VI and VII. These findings are of particular interest because, to date, polyamine values for testicular tissue have not been reported. Putrescine values are not included in the tables,

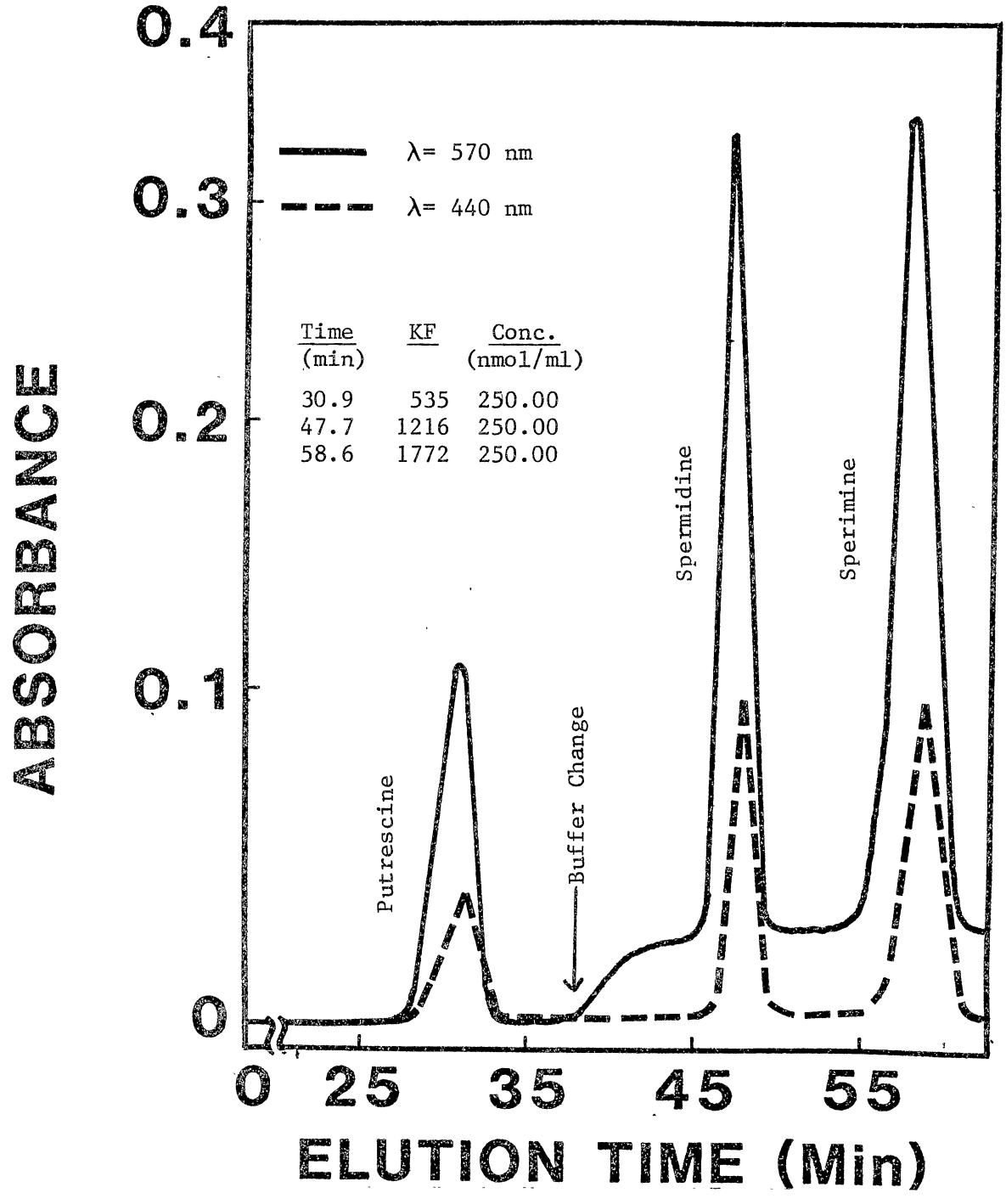


Figure 7. A Chromatogram of Standards by Amino Acid Analysis

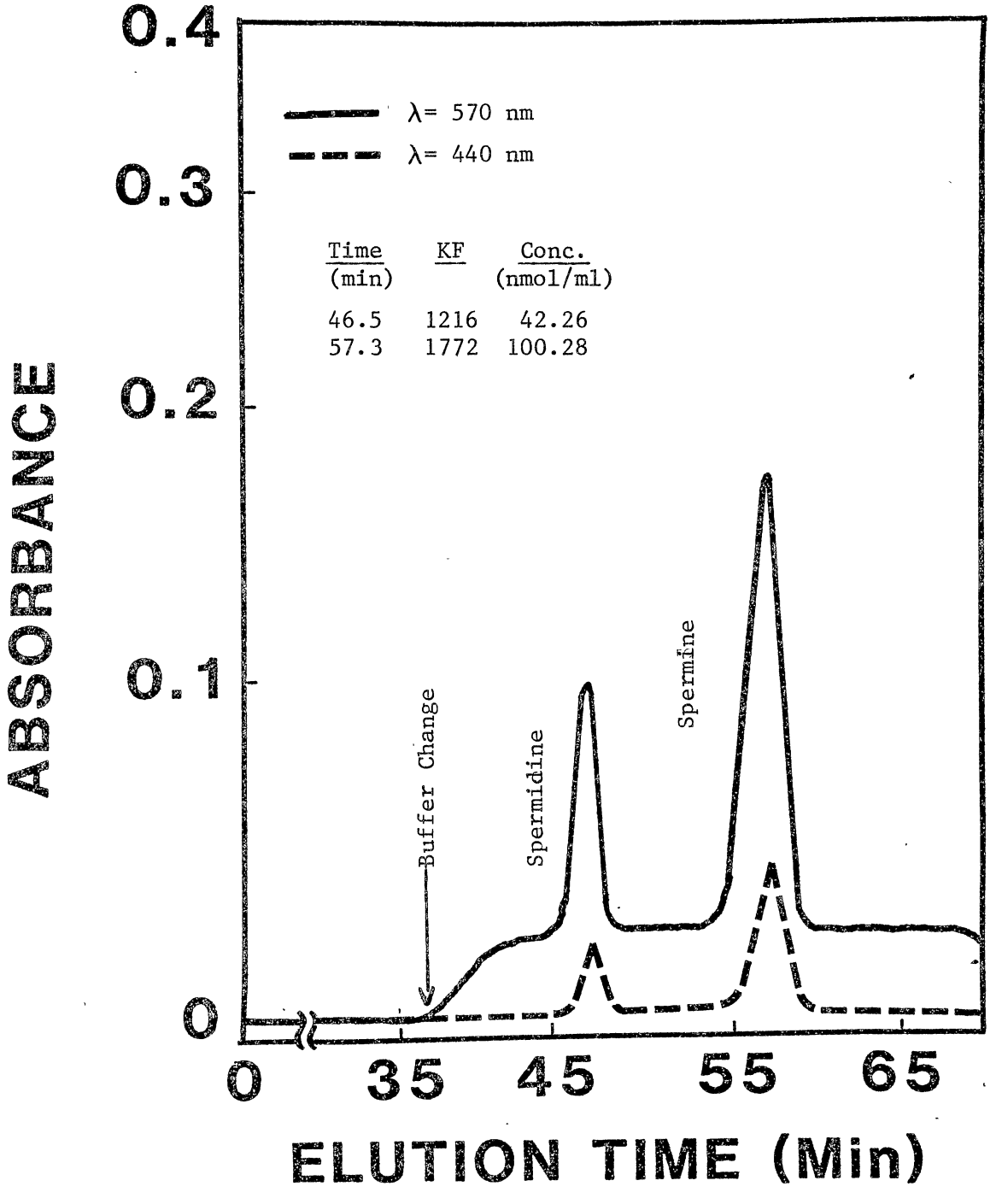


Figure 8. A Chromatogram of Unknown Sample by Amino Acid Analysis

TABLE VI

MEAN POLYAMINE CONCENTRATION ± SD FOR EXPERIMENT I

Age	Diet					
	Vitamin A-Deficient			Retinyl Palmitate-Supplemented		
	n	Spermidine ± SD	Spermine ± SD	n	Spermidine ± SD	Spermine ± SD
	($\frac{nmol}{mg\ DNA}$)			($\frac{nmol}{mg\ DNA}$)		
7 Weeks	3	66.5 ± 7.05	153 ± 26.5	3	72.6 ± 1.53	165 ± 7.81
10½ Weeks	6	74.8 ± 5.95	179 ± 16.0	6	68.5 ± 7.10	180 ± 16.9

	Diet[1]								
	Retinyl Palmitate-Supplemented			Retinoic Acid-Supplemented					
	n	Spermidine ± SD	Spermine ± SD	n	Spermidine ± SD	Spermine ± SD	n	Spermidine ± SD	Spermine ± SD
	($\frac{nmol}{mg\ DNA}$)								
14½ Weeks	6	75.2 ± 5.95	191 ± 2.65	6	154 ± 80.3**	276 ± 111			
18½ Weeks	6	76.6 ± 3.69	183 ± 9.40	6	195 ± 43.7*	318 ± 47.6*	6	70.9 ± 5.35	180 ± 10.6
21½ Weeks	6	72.8 ± 4.26	183 ± 7.77	6	145 ± 32.8*	238 ± 61.2**	3	71.3 ± 7.44	186 ± 14.7

[1]Vitamin A-deficient rats were placed either on retinyl palmitate- or retinoic acid-supplemented diets at 10½ weeks when they reached weight plateau.

*Significantly different from controls at $\alpha = .05$.

**Significantly different from controls at $\alpha = .10$.

TABLE VII

MEAN POLYAMINE CONCENTRATION ± SD FOR EXPERIMENT II

Age	Diet					
	Vitamin A-Deficient			Retinyl Palmitate-Supplemented		
	n	Spermidine ± SD	Spermine ± SD	n	Spermidine ± SD	Spermine ± SD
	($\frac{nmol}{mg\ DNA}$)			($\frac{nmol}{mg\ DNA}$)		
7 Weeks	3	73.6 ± 3.72	168 ± 7.81	3	78.4 ± 5.40	173 ± 9.81
12 Weeks	3	75.4 ± 4.76	189 ± 4.36*	3	79.8 ± 3.35	205 ± 5.51

	Diet[1]								
	Retinyl Palmitate-Supplemented			Retinoic Acid-Supplemented			Retinyl Palmitate-Supplemented		
	n	Spermidine ± SD	Spermine ± SD	n	Spermidine ± SD	Spermine ± SD	n	Spermidine ± SD	Spermine ± SD
	($\frac{nmol}{mg\ DNA}$)						($\frac{nmol}{mg\ DNA}$)		
16 Weeks	3	78.8 ± 6.50	187 ± 9.87	3	90.1 ± 14.7	191 ± 12.9	3	70.3 ± 7.19	176 ± 8.62
19½ Weeks	2	73.4 ± 15.0	165 ± 24.0	3	125 ± 30.3	171 ± 45.5	3	68.3 ± 4.68	166 ± 5.03

[1]Vitamin A-deficient rats were placed either on retinyl palmitate- or retinoic acid-supplemented diets at 12 weeks when they reached weight plateau.

*Significantly different from controls at $\alpha = .05$.

because, with the procedure and equipment utilized, which can detect as little as 0.2 nmol/100 µl, no putrescine was detected in any of the samples.

At seven weeks of age, when the first assay was conducted for Experiment I, both weight and ODC activity of the retinyl palmitate-supplemented control group were significantly different from the animals consuming the vitamin A-deficient diet, as described in detail previously. In contrast, no differences in spermidine or spermine concentrations were noted between the two treatment groups at the time of the first sacrifice for either Experiment I or II. On the other hand, at weight plateau, weight and ODC activity were the same in both Experiment I and II. While spermidine and spermine values of the two dietary treatment groups were not different for Experiment I, spermine concentrations were significantly different for the two groups in Experiment II at weight plateau (189 nmol/mg DNA ± 4.36 for the deficient animals versus 205 nmol/mg DNA ± 5.51 for the retinyl palmitate-supplemented rats). The reason for this difference is unclear. Spermidine values, in contrast, were not different in Experiment II at weight plateau.

After supplementation of the deficient rats with either retinyl palmitate or retinoic acid, no differences between the two control groups (retinyl palmitate throughout the study and retinyl palmitate-supplemented from weight plateau) were found for spermidine or spermine in either experiment. This reinforces the contention that the first weeks of deficiency had no adverse effects on the primary control group (retinyl palmitate-supplemented from weight plateau).

The experimental group exhibited notable differences from controls

in several instances. In Experiment I, the first assay following supplementation (14½ weeks) of the deficient animals showed no significant differences in polyamine values, but a tendency for spermidine concentrations of the retinoic acid-supplemented animals to be greater than the control was established ($P < .10$). By the time the second assay following supplementation (18½ weeks) was conducted, both spermidine and spermine concentrations were significantly greater than their corresponding controls. As for the ODC procedure, the edematous fluid contained in the testes of the retinoic acid-supplemented rats was not included in the polyamine analysis at the time of this assay. The final assay of Experiment I (21½ weeks) included the edematous fluid of the testes. Again, as at 18½ weeks, spermidine values were significantly greater than controls; spermine values of the controls and experimental group were not significantly different, although a definite tendency ($P < .10$) for retinoic acid-supplemented animals to have greater testicular spermine concentrations than controls was noted.

In Experiment II, no significant differences were determined following supplementation of the vitamin A-deficient rats. At the time of the first assay following supplementation (16 weeks), spermidine and spermine concentrations were not different for controls or the experimental group. Likewise, at the time of the second assay following supplementation (19½ weeks), differences were not significant. Although spermine values appear to be the same for all dietary treatment groups, spermidine concentrations appear different for the experimental group versus controls (retinyl palmitate from weight plateau, 73.4 nmol/mg DNA; retinyl palmitate throughout study, 68.3

nmol/mg DNA ± 4.68; retinoic acid from weight plateau, 125 nmol/mg DNA ± 30.3). The difference is not great enough to be statistically significant given the small number of rats per treatment group (retinyl palmitate from weight plateau, n = 2; retinyl palmitate throughout study, n = 3; retinoic acid from weight plateau, n = 2).

The spermidine/spermine ratio is considered an indicator of proliferation by some researchers (Jänne et al., 1964; Neishanakoy, 1968; Jänne et al., 1974). Therefore, this information was gathered and the results are displayed in Figures 9 and 10. As can be seen, the ratio of the retinoic acid-supplemented rats increases markedly, while that of the controls remains fairly constant. Hence, it appears that as the testes degenerate due to retinol deficiency, the spermidine/spermine ratio increases.

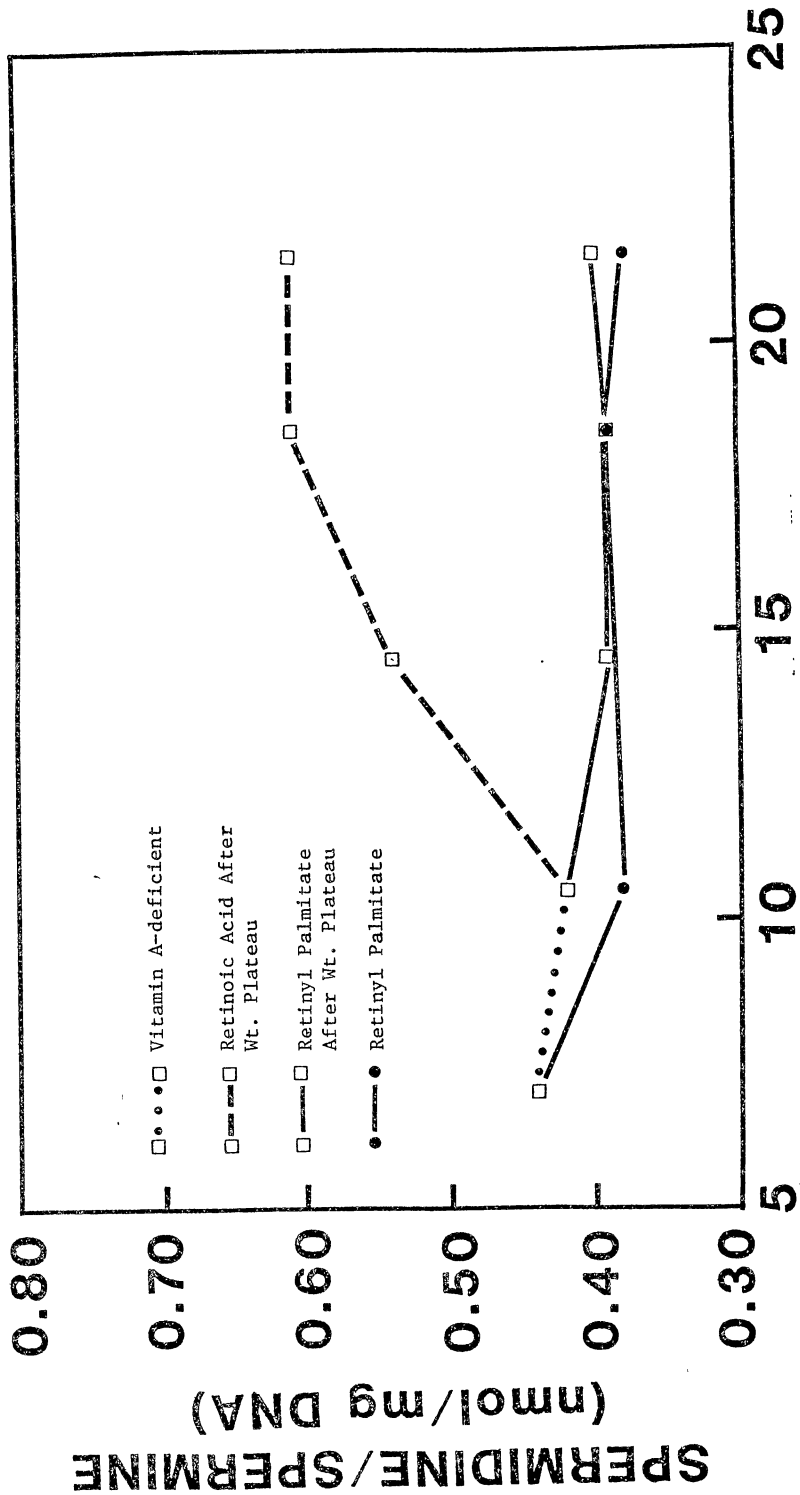


Figure 9. Mean Spermidine/Spermine Ratio in Rat Testes: Experiment I

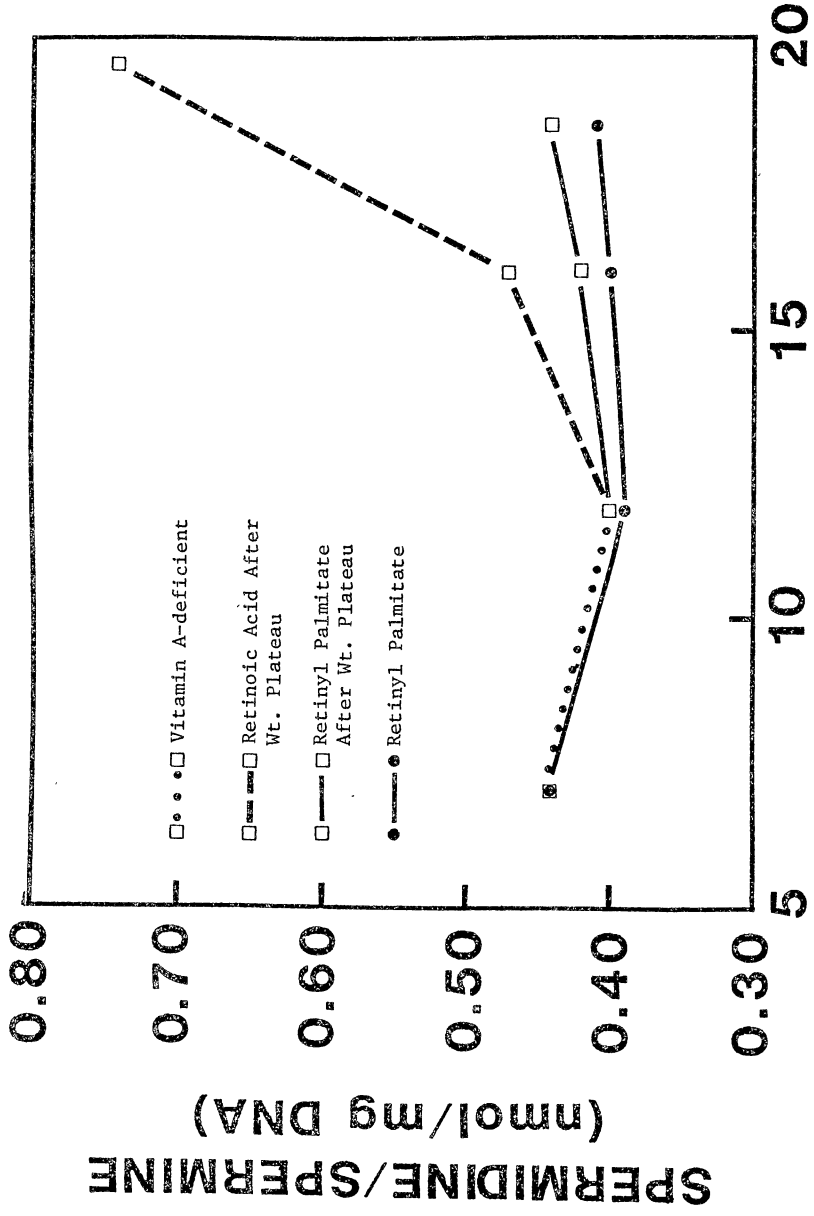


Figure 10. Mean Spermidine/Spermine Ratio in Rat Testes: Experiment II

CHAPTER IV

DISCUSSION

The results of this study suggest that vitamin A exerts an effect on the activity of ornithine decarboxylase and polyamine levels in rat testes. By eliminating retinol from the diet of rats, it was possible to induce progressive degeneration of testicular tissue. In Experiment I, the degenerating testes of retinol-deficient, retinoic acid-supplemented rats had significantly greater levels of ODC activity than the levels found in rats maintained on a retinyl palmitate-supplemented diet. The mechanism responsible for these findings cannot be determined from these experiments, but the data do provide valuable information for future studies.

The hypothesis proposed by Russell and colleagues (Russell et al., 1976), discussed in Chapter I, involves a cascade of events leading up to the transamidation of putrescine to glutamine residues of ODC by transglutaminase. By this model, once ODC is conjugated with putrescine, it can no longer function as a decarboxylase. Rather, the complex serves as subunit s3 of RNA polymerase I, rendering the polymerase active, and consequently enhancing RNA production, and evantually, protein synthesis. Evidence cited in recent reports has shown that transglutaminase may be modified by retinoids (Yuspa et al., 1982; Scott et al., 1982). Their studies demonstrate that both retinol and retinoic acid can induce transglutaminase in various

tissues. Since the testis is unique, in that degeneration occurs in the absence of retinol despite the presence of retinoic acid, it is conceivable that testicular transglutaminase may be regulated by retinol. In that case, ODC may no longer conjugate with putrescine in retinol-deficient testes and the ODC-putrescine complex could not serve as the RNA polymerase subunit. One would therefore expect a decrease in RNA synthesis. This has been established by Chaudhary (1974) who showed that testicular RNA decreased significantly in rats maintained on a retinol-deficient, retinoic acid-supplemented diet as compared to controls fed retinyl acetate. In short, Russell et al. (1976) contend that normally, ODC fulfills two functions: 1) as the rate-limiting enzyme of the polyamine biosynthetic pathway and 2) as s3 of RNA polymerase I. The results of the present study suggest that in the absence of retinol ODC can function only as the decarboxylase, thereby explaining the elevated ODC activity observed in the retinol-deficient, retinoic acid-supplemented animals, since the vast majority of ODC would be utilized in polyamine biosynthesis. This explanation also supports the concept that vitamin A is involved in gene expression (Omori and Chytil, 1982). In addition, higher polyamine concentrations in deficient testes than controls would be an expected consequence of the elevated ODC activity. In fact, increased concentrations of spermidine and spermine are observed.

Included in the above model is a cyclic AMP-dependent protein kinase necessary for the phosphorylation of proteins associated with the ODC gene. Again, retinoids have been implicated by Scott et al. (1982) who contend that an alternative mode of action for the retinoids may be the regulation of protein kinase translocation from the cytosol

to the nucleus. Based on the results of the present study, if the protein kinase specifically required retinol for translocation, then one could speculate that in the absence of retinol, the transcription of ODC would not be enhanced, so less ODC would be available for polyamine biosynthesis and conjugation with putrescine. Hence, one would expect lower ODC activity and polyamine levels, which contradicts the results of the present study. Therefore, based on the findings of this study, retinoid-controlled translocation of protein kinase seems unlikely.

Atmar and Kuehn (1981) have also proposed a complex model for ODC regulation. They hypothesize that a polyamine-dependent protein kinase phosphorylates ODC. In this phosphorylated form, ODC can bind with genes coding for ribosomal RNA and stimulate ribosomal RNA synthesis through action on RNA polymerase I. Again, vitamin A might fit into this scheme if the protein kinase required retinol in addition to polyamines in the testes. This is purely speculation, since no evidence to date confirms the link between vitamin A and protein kinase. However, in the event that the kinase did require retinol, the retinol-deficient testes would exhibit higher ODC activity than controls because, as in the Russell model, more ODC would be available for the decarboxylase function, since the absence of retinol would not permit phosphorylation of the enzyme; consequently, ribosomal RNA synthesis would not be stimulated.

Vitamin A is known to be involved in cell growth and cellular differentiation (Wohlbach and Howe, 1925). Studies indicate that during liver regeneration, polyamine levels and ODC activity are elevated but return to normal levels when regeneration is complete

(Russell and Snyder, 1968). Furthermore, Schinder et al. (1983) report that ODC inhibition induces differentiation in embryonal carcinoma cells. In the case of the retinyl palmitate-supplemented rats, testes development and spermatogenesis proceed to the point of terminal differentiation. On the other hand, the retinol-deficient testes never reach terminal differentiation; the formation of spermatozoa. It may be that whatever mechanism induces ODC activity and increases polyamine levels during active growth and differentiation of normal testes, later reduces ODC activity and lowers polyamine levels once the normal process of maintenance of spermatogenesis begins (Osterman et al., 1983). If that were the case, the mechanism would never be triggered to slow down in the animals consuming the retinol-deficient, retinoic acid-supplemented diet. This would be compatible with the results of this study; namely, elevated levels of ODC and the polyamines (spermidine and spermine) in rats consuming a retinol-deficient, retinoic acid-supplemented diet.

Another model for ODC regulation involves the formation of a so-called antizyme complex. This complex reportedly inhibits and regulates the activity of ODC (Fong et al., 1976; Heller et al., 1976; Heller et al., 1983). Fujita et al. (1982) proceed one step further with this theory. They have discovered a macromolecular inhibitor of the antizyme which serves to re-activate ODC. These researchers suggest that the antizyme inhibitor has a greater affinity for the antizyme than does ODC and therefore can replace ODC in the complex. The presence of an antizyme and an antizyme inhibitor is indicative of a very sensitive control mechanism for the regulation

of ODC. The polyamines have already been shown to induce the antizyme (Heller et al., 1976). It is not inconceivable that vitamin A also fits into this scheme. An interesting area for future research would be to investigate a possible role for vitamin A in this mechanism.

The spermidine/spermine (nmol/mg DNA) ratios in testes of controls in this study are in agreement with the value reported for testicular tissue by Jänne et al. (1964), 0.44 ± .04 (SD). At the time of the first two assays, the rats consuming the vitamin A-deficient diet also had ratios near the value reported by Jänne et al. However, after the animals reached a weight plateau, a gradual increase in the ratio was observed. Both spermidine and spermine values increased after animals were supplemented with retinoic acid, but the elevation of spermidine was more pronounced than that of spermine. Because ODC activity increased in the retinol-deficient testes of experimental animals, one would expect elevated polyamine levels. Surprisingly, no putrescine was detected. There are at least two explanations for this absence: 1) The method used in these experiments could not detect less than 0.2 nmol. It is possible that the diamine was present in quantities less than 0.2 nmol. 2) S-adenosyl-L-methionine decarboxylase (SAMD) which supplies the aminopropyl group for the formation of spermidine and spermine from putrescine may be more active in rat testes and thereby prevent the accumulation of putrescine.

Both spermidine and spermine concentrations increased with age in those rats consuming the retinol-deficient, retinoic acid-supplemented diet. However, spermidine levels increased more than spermine levels. The reason for this is unclear, but recent evidence (Oka et al., 1982)

has demonstrated a need for spermidine in the production of milk protein by the cultured mouse mammary gland. In the testis deficient in retinol, differentiation ceases. Spermidine might subsequently accumulate.

In summary, the results of this study provide evidence that vitamin A does play a role in the regulation of ODC activity and polyamine levels. Although no conclusions can be drawn regarding the nature of that role, several areas have been explored. In addition, possibilities for further research, based on the findings, have been presented and discussed.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objective of this study was to determine if vitamin A is linked to ODC regulation and/or the polyamine biosynthetic pathway in rat testes. Rats were placed on one of three diets: 1) Vitamin A-deficient until weight plateau, after which animals were supplemented with retinoic acid. 2) Vitamin A-deficient until weight plateau after which animals were supplemented with retinyl palmitate. 3) Retinyl palmitate-supplemented throughout the study. Assays were conducted periodically. ODC specific activity and polyamine concentrations were measured. The retinoic acid-supplemented rats exhibited significantly greater ODC activity and spermidine and spermine levels than either group supplemented with retinyl palmitate in their diet. Furthermore, the spermidine/spermine (nmol/mg DNA) ratio of retinoic acid-supplemented rats increased with age and progressive tissue degeneration. No putrescine was detected in any of the testes (minimum detectable was 0.2 nmol).

In the present study, vitamin A influenced ODC activity and polyamine concentrations. The results of this study are compatible with several models postulating ODC regulation and polyamine biosynthesis and control. Although the exact mode of action was not defined, this study opens up a number of areas for future research.

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APPENDIX A

TABLE VIII

VITAMIN A DEFICIENT PURIFIED DIET FOR RATS*

Ingredient	g/Kg
Casein, "Vitamin-Free" Test	193.0
DL-Methionine	3.0
Corn Starch	665.1343
Cottonseed oil	50.0
Fiber (cellulose)	50.0
Mineral Mix, AIN-76**	35.0
Biotin	0.0004
Vitamin B_{12} (0.1% trituration in mannitol)	0.0297
Calcium Pantothenate	0.0661
Choline Dihydrogen Citrate	3.4969
Folic Acid	0.002
Menadione Sodium Bisulfite Complex	0.05
Niacin	0.0991
Pyridoxine HCl	0.022
Riboflavin	0.022
Thiamin HCl	0.022
DL-Alpha Tocopheryl Acetate (1000 U/g)	0.05
Vitamin D. in corn oil (400,000 U/g)	0.0055

* Prepared for us by Teklad, Madison, WI.

** J. Nutr. 107: 1340-1348 (1977).

VITA 2

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Master of Science

Thesis: THE EFFECT OF DIETARY RETINYL PALMITATE AND RETINOIC ACID ON ORNITHINE DECARBOXYLASE ACTIVITY AND POLYAMINE CONCENTRATION IN RAT TESTES

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