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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE ROLE OF RETINOIC ACID IN THE SYNTHESIS OF RAPIDLY LABELED NUCLEAR RIBONUCLEIC ACID

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

NAOKI CHIBA

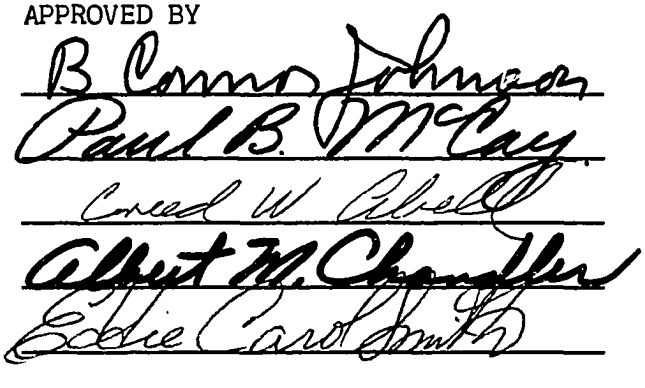
Oklahoma City, Oklahoma

1970

THE ROLE OF RETINOIC ACID IN THE SYNTHESIS OF

RAPIDLY LABELED NUCLEAR RIBONUCLEIC ACID

APPROVED BY



DISSERTATION COMMITTEE

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TO

AKIKO

MAKI, KAYO and an Expected Child

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THE ROLE OF RETINOIC ACID IN THE SYNTHESIS OF RAPIDLY LABELED NUCLEAR RIBONUCLEIC ACID

CHAPTER I

INTRODUCTION

Vitamin A, one of the earliest vitamins to be discovered, is an absolute nutritional requirement for life in almost all animal species. On the other hand, there is apparently no nutritional nor metabolic requirement of vitamin A or its derivatives by microorganisms, plants nor insects. This observation alone indicates that vitamin A possibly functions in the specific regulation of the complex animal. However, vitamin A must be obtained from the diet rather than by synthesis in the animal body. The specificity of target organs, e.g., epithelial cells, as well as species specificity indicates a highly specialized role for as vitamin A as compared to the B vitamins which appear to function as precursors of coenzyme compounds essential to all life.

Dietary provitamins and vitamin A are sources of tissue deposits of retinol, retinyl esters, retinal and retinoic acid. Retinyl esters and retinol constitute a storage and transport system (1) which allows a continuous supply of the active form of the vitamin to whatever site is required. 11-Cis-retinal is the active form of vitamin A in vision, being the prosthetic group of the visual pigments (2). The role of vitamin A

in vision has been reviewed (3). Retinoic acid and its esters were first obtained during attempts at the chemical synthesis of retinol (4). The finding that retinoic acid had relatively high biological activity in rat growth led to the speculation that retinoic acid was a metabolite of retinol, and was a precursor of, or was itself, the systemically active form of vitamin A (5). Attempts to trap radioactivity in unlabeled retinoic acid after administration of ^{14}C-retinol have not been successful (6, 7). Failure to detect retinoic acid may be due to its rapid metabolism; also much of the retinal given to animals would be stored as retinyl esters and would, therefore, be protected from oxidation. Malathi et al. (8) reported that retinoic acid was 141% as active as retinyl acetate in a series of experiments to measure the changes in body weight in vitamin A-deficient rats given retinoic acid intraperitoneally. The formation of retinoic acid in vivo, essential for the normal biological action of retinol has not yet been proved. Experimental evidence is, however, compatible with the view that retinoic acid is a normal precursor of the active forms of vitamin A (9).

Although the role of vitamin A in vision is important, this is not its only function, since an animal can die from vitamin A deficiency but not necessarily from blindness. What is the role of vitamin A in metabolism? How does the vitamin act at the molecular level in the tissue cells? Numerous attempts have been made to establish the nutritional role of vitamin A in the animal body. Although much effort and research have been extended in the field of the morphological changes caused by vitamin A deficiency such as epithelial keratinization, the cause of such morphological changes has not been established at the molecular level.

At the present time, the biological mechanism of vitamin A action has not been established. However, the findings from the following studies on the role of vitamin A in nutrition may suggest that the site of action of vitamin A in the tissue cells is in the regulation of specific protein synthesis (10). If this is true, this regulation may take place either at the transcription level, i.e., in RNA synthesis, or at the translation level, i.e., in protein synthesis.

1) Using ^{14}C-labeled acetate, lactate, glycerol and glucose it was found that the conversion of acetate, lactate and glycerol but not glucose to liver glycogen was blocked by vitamin A deficiency using pair-fed controls (11, 12). Corticosterone completely reversed this block although it did not cure the gross symptoms of vitamin A deficiency. On this basis it was proposed that the deficiency caused some adrenal change interfering with glucocorticoid production. A number of different sites of function of vitamin A has been proposed with regard to steroid hormones (11, 13-17). Treatment of the vitamin A-deficient rat with corticosterone or cortisone did not alter the deficiency (11) and even made it worse (18). This suggested that vitamin A deficiency resulted in a nutritional destruction of adrenal cortex function even though little change could be seen by analytical methods.

2) The possible nutritional role of vitamin A in the biosynthesis of mucopolysaccharide (MPS) in the rat has been extensively examined. Vitamin A deficiency lowered the incorporation of ^{35}S-sulfate and ^{14}C-glucose into intestinal MPS (19, 20, 21). However, some workers have reported no effect of vitamin A deficiency on this reaction (22, 23) and this has recently proved to be an inanition effect (24, 25).

3) Vitamin A has been implicated with the functioning of those membranes involved with NADPH mixed function oxidation reactions (13, 26, 27). A 30 to 70% reduction in liver microsomal codeine demethylase activity was found which varied as a function of the deficiency (28). It was also found that the marked increase in the activity of the enzyme brought about by phenobarbital or codeine was inhibited by vitamin A deficiency (29) and that the cure by vitamin A within 48 hours as measured by increased codeine demethylase activity was inhibited by actinomycin D (30). Again this has recently been shown to be an inanition effect (31).

4) The early literature on the effect of vitamin A nutrition on differentiation and structure of epithelial mucosal cells has been reviewed (32). It was reported that vitamin A completely suppressed keratinization in cultured skin cells from 9 day old chick embryos (33). Vitamin A also has been reported to inhibit squamous changes in skin cancers induced by chemical agents (34, 35). Palludan suggested that the effect of vitamin A on the testes is direct and not via hormone production (36).

5) De Luca *et al.* have found that certain aminoacyl-t-RNA synthetases of the intestinal mucosa are decreased by vitamin A deficiency (10). From this finding they concluded that protein synthesis by membrane-bound but not by free polyribosomes of intestinal mucosa was depressed under conditions of vitamin A deficiency and that vitamin A is therefore involved, directly or indirectly, in protein synthesis at the translation level. They also suggested that vitamin A has a regulatory role in specific protein synthesis.

6) Zachman has reported that retinol administration stimulated

the *in vivo* incorporation of labeled uridine into total intestinal mucosal RNA of the vitamin A-deficient rat, but that it had no effect on the rate of incorporation of labeled uridine into liver or kidney RNA (37).

From the work described above, it appeared that a site to examine in searching for the role of vitamin A is in the regulation of synthesis of specific protein and thus control of cell structure and differentiation. Since specific proteins are molecules that confer specificity of function and/or morphology and since this regulation might most probably take place at the transcription level, i.e., in RNA synthesis, this was the site examined.

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Weanling male albino rats of the Holtzman and Sprague-Dawley strains were maintained on vitamin A-normal or A-deficient diets as described below (38). All animals were watered with distilled water.

Vitamin A deficiency usually developed 5 to 8 weeks after maintaining the animals on the vitamin A-deficient diet. The body weights of the deficient rats averaged 30 to 50 grams less than the normal rats.

Diet Materials

The composition of the vitamin A normal diet is shown below. The vitamin-free casein, sucrose, NRL salt mixture, choline chloride, L-cystine, Alphacel, dextrose, and vitamin D (Calciferol) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Sigma Chemical Company, St. Louis, Missouri, provided the remaining vitamins. The sucrose was purchased from Collin and Dietz, Wholesalers and the cottonseed oil came from a local manufacturer.

Diet Composition

Ingredient	per Kg diet
Vitamin-free casein (heated)	240.0 g
Sucrose	645.0 g
Salt mix 446	50.0 g
Heat-treated cotton seed oil	30.0 g
Choline citrate	3.0 g
Vitamin premix (described below)	10.0 g
Cysteine	2.0 g
Ascorbic acid	0.1 g
Alphacel	20.0 g

The composition of the vitamin premix was as follows:

Ingredient	per 100 g
Thiamine-HCl	0.25 g
Riboflavin	0.25 g
Calcium panthothenate	0.80 g
Nicotinic acid	1.50 g
Pyridoxine-HCl	1.50 g
Biotin	0.01 g
Folic acid	0.05 g
Vitamin B_{12} (0.1% in mannitol)	0.20 g
Menadione	0.02 g
Alpha-tocopheryl succinate	6.0 g
Vitamin D (Calciferol)	0.04 g (2,000 I.U.)
Vitamin A palmitate	0.80 g (20,000 I.U.)
Dextrose	89.58 g

For the vitamin A-deficient diet the vitamin A palmitate was eliminated from the vitamin premix and supplemented with dextrose to make 100 g vitamin premix.

Reagents

Potassium retinoate was prepared by dissolving a given amount of retinoic acid which was obtained from Hoffmann-La Roche & Company, Basle, Switzerland, in 0.2 N potassium hydroxide solution, adjusting to pH 7.5 with a 20% potassium acetate solution that had been adjusted to pH 5.0 with glacial acetic acid. This solution was diluted with normal saline to make up the desired concentration.

Retinol obtained from Hoffmann-La Roche & Company was dissolved in absolute ethyl alcohol and diluted with deionized water to make a 50% ethanol solution. Tritium labeled retinoic acid (vitamin A -10,11-$^{3}H_{2}$-acid) was obtained from Hoffmann-La Roche & Company. A solution containing 41.05 mg (= 14.46 mCi) in toluene was prepared having a specific activity of 352 μCi per mg.

Tritium labeled uridine (uridine-5-^{3}H) was obtained from New England Nuclear Corporation, Boston, Massachusetts. Different lots contained from 0.012 to 0.015 mg uridine per mCi. Labeled uridine was dissolved in sterile normal saline to an initial concentration of 100 μCi per 0.5 ml and diluted when required, with normal saline, to a final concentration of 5 or 10 μCi per 0.5 ml for injection. This sterile normal saline contained 0.9 g of sodium chloride, U.S.P. in 100 ml and was obtained from Cutter Laboratories, Berkeley, California.

Tritium labeled thymidine was obtained from New England Nuclear Corporation, Boston, Massachusetts. Five μCi of thymidine-^{3}H per 0.5 ml

per animal was used.

NCS-Reagent obtained from Nuclear-Chicago Corporation, Des Plaines, Illinois, was used as a solubilizer for tissue homogenate for liquid scintillation counting (39). The composition of the scintillation fluid used was as follows: PPO...10 g, POPOP...0.5 g, Naphthalene...80 g, Xylene...143 ml, p-Dioxane...429 ml and Ethylene glycol monoethyl ether ...429 ml. The following chemicals were obtained from Packard Instrument Company, Downers Grove, Illinois: PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene.

Sucrose used for sucrose density gradient centrifugation was obtained from Mann Research Laboratories, New York, N. Y., and was free from ribonuclease.

Ribonucleic acid from Torula yeast, Grade VI, and deoxyribonuclei acid from calf thymus, sodium salt, highly polymerized, Type I, were also obtained from Sigma Chemical Company and were used to make standard RNA and DNA solutions. Ribonuclease-A from bovine pancreas, 5 x crystallized, type 1-A, was obtained from Sigma Chemical Company, St. Louis.

Extraction buffer was made up as follows: 0.3% sodium deoxycholate and 0.1% polyvinyl sulfate in 0.03 M phosphate buffer containing 0.14 M sodium chloride, pH 6.8.

The following chemicals were obtained from Fisher Scientific Company, Philadelphia, Pennsylvania: sodium chloride, sodium acetate, sodium hydroxide, potassium chloride, potassium acetate, potassium hydroxide, tri (hydroxymethyl) amino methane, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, trichloroacetic acid, copper chloride, magnesium chloride, orcinol, xylene, sodium deoxycholate and lique-

fied phenol which were all reagent or analytical grade.

Naphthalene was obtained from Eastman Kodak Company, Rochester, New York.

Polyvinyl sulfate was obtained from K & K Laboratories, Inc., Plainview, New York.

P-dioxane, ethyl oxide, ethylene glycol monoethyl ether and isoamyl alcohol were obtained from Baker Chemical Company, Phillipsburg, New Jersey.

Pure U.S.P. ethyl alcohol was obtained from U.S. Industrial Chemicals Co., New York, New York.

Instruments and Equipment

Spectrophotometric measurements were made either using a Beckman DU-2 from Beckman Instruments Company, South Pasadena, California, or a Gilford Model 240 from Gilford Instrument Laboratories, Inc., Oberlin, Ohio. Centrifugations were carried out with an International Refrigerated Centrifuge, International Equipment Company, Boston, Massachusetts, a Sorvall Automatic Refrigerated centrifuge, RC 2-B, Ivan Sorvall, Inc., Norwalk, Connecticut and a Spinco Model L2-65 Ultracentrifuge, Beckman Instruments Company, Spinco Division, Palo Alto, California. The water bath used was Metabolyte Water-Bath Shaker with temperature control, Model G 77, New Brunswick Scientific Company, Inc., New Brunswick, New Jersey. Radioactivity was measured with a Mark I, Liquid Scintillation Counter, Nuclear-Chicago Corporation, Des Plaines, Illinois.

Methods

Total RNA Isolation

Total RNA was isolated by a method modified from that of Lang and Sekeris (40). Individual rat liver (average weight of vitamin A-deficient rat liver, 4 g) was homogenized in 20 ml of extraction buffer using a Potter-Elvehjem glass homogenizer. The homogenate was transferred to a flask containing 20 ml 80% phenol and extracted for 30 minutes at 65°C with vigorous shaking. Twenty milliliters 0.9% saline was added and the suspension centrifuged for 30 minutes at 15,000 rpm. The upper aqueous layer was saved. The interphase and lower phenol phase were treated with an additional 20 ml extraction buffer for 15 minutes at 65°C with vigorous shaking. Centrifugation was carried out as before and the two supernatants were combined and shaken with 20 ml 80% phenol for 15 minutes at room temperature. After separating the aqueous phase from the phenol phase by centrifugation, the aqueous phase was washed at least three times with an equal volume of peroxide-free diethyl ether. Residual ether was removed by passing nitrogen gas through the solution. One drop of 10% sodium chloride solution was added per 2 ml of the aqueous solution. The RNA was precipitated by adding two volumes of 90% ethyl alcohol and storing the solution in a freezer overnight.

The RNA obtained was purified as follows: The RNA was collected by centrifugation and dissolved in 0.01 M sodium acetate buffer, pH 5.1, containing 0.1 M sodium chloride, precipitated by addition of two volumes of ethyl alcohol and stored in a freezer for at least one hour. This procedure was repeated. The purified RNA precipitate was dissolved in 20 ml of the 0.01 M sodium acetate buffer, pH 5.1.

Isolation of Liver Nuclei

Nuclei were isolated using the method of Widnell and Tata (41). Livers of two to four rats were pooled for each isolation. All procedures were performed at 0° to 4°C. The tissue was rinsed once with ice-cold saline and minced with a pair of scissors in three volumes of 0.32 M sucrose solution containing 3 mM magnesium chloride (one part liver, three parts medium). The minced liver was homogenized in a Potter-Elvehjem glass homogenizer. The homogenate was filtered through two layers of cheese cloth and the filtrate was diluted to 0.25 M with ice-cold deionized water. The sucrose solution was centrifuged at 2,000 rpm for 10 minutes. The supernatant was saved for the isolation of cytoplasmic RNA. The nuclear pellet thus obtained was purified as follows: it was suspended in 2.4 M sucrose solution containing 0.001 M magnesium chloride with the aid of a homogenizer and centrifuged in a Spinco No. 30 rotor at 25,000 rpm for 60 minutes. In some experiments this purification step was repeated.

Liver Nuclear RNA Isolation

The isolated nuclei (approximately 0.1 g) were treated according to the method of Lang and Sekeris (40) as follows: the nuclei obtained were homogenized in a 20 ml extraction buffer; the homogenate was shaken with 10 ml 80% phenol for 15 minutes at 50°C and centrifuged at 15,000 rpm for 30 minutes. For sedimentation in sucrose density gradients, the aqueous supernatant obtained from the first centrifugation was combined with the second supernatant after extraction at 65°C. However, for other experiments the first supernatant was treated separately. A second supernatant was obtained by extracting the interphase and phenol

phase with another 20 ml extraction buffer for 15 minutes at 65°C. The supernatant was treated exactly as in the total RNA isolation procedure described above. The nuclear RNA thus obtained is reported to be high in messenger activity (40).

RNA Isolation from the Small Intestine

The small intestine was quickly removed from the body and washed with cold saline, both inside and outside. The small intestine was stretched out on an iced glass plate in a cold room and laid open longitudinally with a pair of scissors. The mucosal membranes were scraped off on the iced glass plate by means of a 25 x 75 mm micro slide and homogenized in a Potter-Elvehjem homogenizer.

RNA Isolation from Kidney

Kidney was first roughly homogenized by means of a small size Waring Blender and then more thoroughly dispersed using a Potter-Elvehjem glass homogenizer. After homogenization, the isolation steps were the same as those described above for liver RNA.

DNA Isolation from Liver and Intestinal Mucosal Membranes

After the nuclei were isolated according to the method described above (41), DNA was isolated using the "hot-salt" method of Niehaus and Barnum (42) and purified and solubilized by the method of Chandler and Neuhaus (43) to count the radioactivity and to determine the amount of DNA present in the solution. The nuclear pellet was dispersed in 10% sodium chloride in 0.4 M Tris-HCl buffer, pH 7.8, incubated at 80°C for 30 minutes and centrifuged. The supernatant was treated by the addition

of two volumes of 95% ethyl alcohol and centrifuged. The precipitate was treated with 0.1 N sodium hydroxide solution at 80 to 90°C for 30 minutes to hydrolyze the RNA, cooled in an ice-bath. Trichloroacetic acid was added to make a 5% solution and centrifuged. The last step was repeated again. The precipitate was washed with absolute ethyl alcohol twice and then with ether. The DNA thus obtained was dissolved in 0.5 M perchloric acid solution and heated at 80°C for 30 minutes. One milliliter of this solution was used for the radioactivity determination and 0.2 ml of the solution was used for the DNA assay.

Isolation of Microsomal RNA from Liver

The microsomal pellet was obtained from the cytoplasmic portion after separating the nuclei, followed by centrifugation at 25,000 rpm for 60 minutes in a Spinco No. 30 rotor of Model L Ultracentrifugation. The microsomal pellet was treated to obtain microsomal RNA in a manner similar to the liver nuclear RNA isolation described above.

Isolation of Transfer RNA from Liver

Transfer RNA was isolated from the supernatant after removal of the microsomal pellet. Further the pH of this supernatant was adjusted to 5.0 with 0.01 M acetic acid solution, and allowed to stand for a few minutes to settle the precipitate. This pH 5 fraction was treated according to the method of Takanami (44) to obtain transfer RNA.

RNA Determination

The Orcinol method of Ceriotti (45) was used to determine the amount of RNA present in the final solution.

DNA Determination

The amount of DNA was determined by means of the diphenylamine procedure of Burton (46).

Administration of Retinoic Acid to the Rat

Retinoic acid was administered to the animals either intraperitoneally or intravenously via the jugular vein as potassium retinoate. Injections were 0.5 ml per rat regardless of the amount of potassium retinoate given.

Sucrose Gradient Resolution

The RNA solutions were purified several times before sedimentation on a linear sucrose density gradient. Sucrose solutions were made up in 0.01 M sodium acetate buffer, pH 5.1, containing 0.1 M sodium chloride. Gradients were prepared by means of a mixer according to the method of McConkey (47). The tubes were kept at 0 to 4°C for 4 to 5 hours before use to enable a limited amount of diffusion between layers to take place. RNA was dissolved in the sodium acetate buffer, pH 5.1, and any insoluble materials were removed by centrifugation. 0.3 to 0.8 ml of RNA solution (containing 0.5 to 1.0 mg RNA/ml) was layered on top of a 5 to 20% linear sucrose density gradient and centrifuged in a Spinco SW-25.1 swinging bucket rotor at 22,500 rpm for 22 hours, or at 25,000 rpm for 17 hours. At the end of the run the rotor was allowed to slow down and come to a stop without the use of the brake. Following centrifugation, the fractions were collected continuously by means of a siphon, the tip of which reached to the bottom of the centrifuge tube. This siphon was connected to a flow-through cell of a Gilford spectrophotometer to meas-

ure the absorbance of the fractions at 260 mμ. Each fraction (1 ml) was collected in a tube after the measurement of the absorbance and then mixed in 15 ml of the scintillation liquid for the counting of radioactivity.

Ribonuclease Digestion

Some RNA samples were treated with ribonuclease prior to sucrose density gradient centrifugation. This ribonuclease treatment consisted of incubating 1.0 ml of a solution of RNA in sodium acetate buffer, pH 5.1, with 5 μg of ribonuclease at 37°C for 10 minutes.

CHAPTER III

RESULTS

Total Liver RNA

In order to determine the effect of vitamin A on RNA synthesis and metabolism, initial experiments were undertaken to study the effect of vitamin A on the incorporation of labeled uridine into total liver RNA. Table 1 shows the results of the first experiment in which labeled uridine was administered to vitamin A-deficient rats and to deficient rats treated with 10 mg (approximately 30,000 International Units of vitamin A activity) of potassium retinoate. From this table it can be seen that deficient rats given potassium retinoate 22 hours before killing incorporated considerably more uridine than did deficient rats not given potassium retinoate.

Figure 1 shows the effects of potassium retinoate given by intraperitoneal injection to deficient rats. The data indicate a marked increase in incorporation of uridine into total liver RNA up to 30 hours after administration of 10 mg potassium retinoate.

In rate studies of uridine incorporation into normal rats (Figure 2) it appears that the peak incorporation occurs about 1 hour after intraperitoneal injection of labeled uridine. However, an increase in RNA specific activity occurs between 2 and 8 hours or later when the

TABLE 1

DIFFERENCES IN RATE OF INCORPORATION OF ^{3}H-URIDINE INTO LIVER RNA OF VITAMIN A-DEFICIENT RATS AND RETINOIC ACID INJECTED-DEFICIENT RATS

Number of Vitamin A-deficient Rats	Potassium Retinoate Injection, mg/rat	^{3}H-Uridine Injection 8.2 Hr after Potassium Retinoate Injection μCi/rat	Time, ^{3}H-Uridine Injection to Sacrifice, hours	dpm/mg RNA
3	10	100	13.6	26,450
3	0	100	13.6	16,100

Three rat livers were pooled.

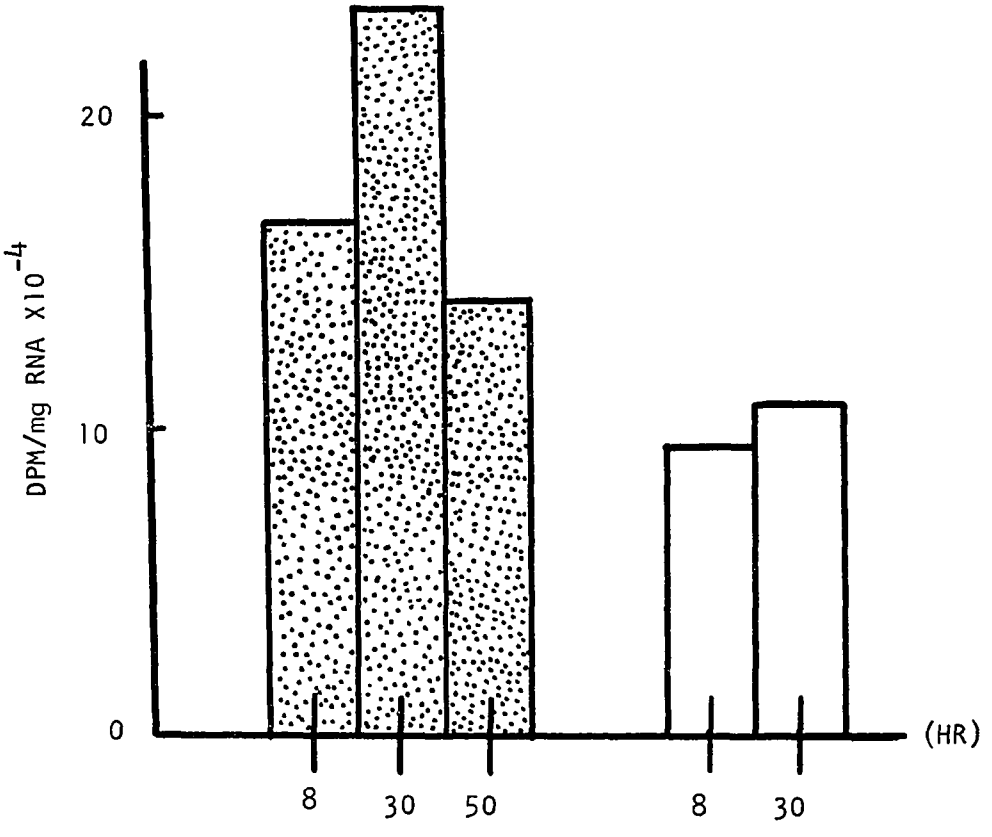


Figure 1. Effect of time from ^{3}H-uridine injection to sacrifice on rate of incorporation of ^{3}H-uridine into total liver RNA of vitamin A deficient rats.

One hundred microcuries of ^{3}H-uridine were injected intraperitoneally per rat at 0 time. Four rat livers were pooled to obtain each point.

▨ Ten milligrams of potassium retinoate were injected intraperitoneally into each rat 1.5 hours before the uridine injection.

▭ No potassium retinoate treatment.

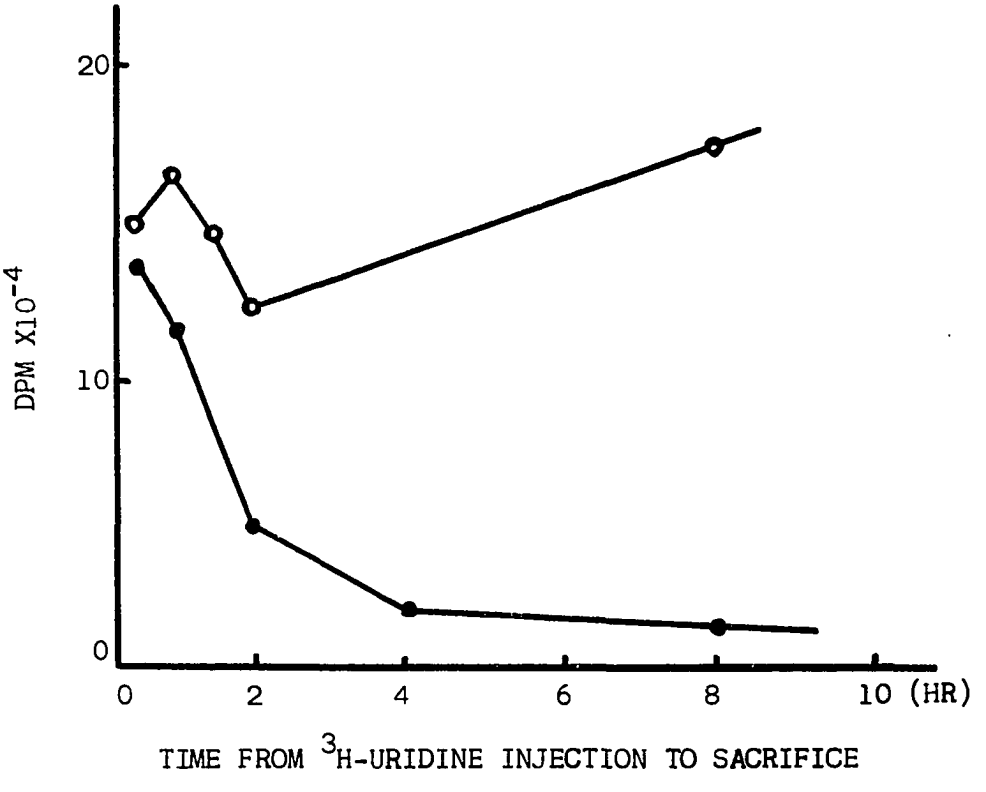


Figure 2. Effect of time from ^{3}H-uridine injection to sacrifice on rate of incorporation of ^{3}H-uridine into total RNA of normal rat liver.

One hundred microcuries of ^{3}H-uridine were injected intraperitoneally into each rat at 0 time. Four rat livers were pooled to obtain each point.

O——O Radioactivity (DPM) per mg RNA.

●——● Radioactivity (DPM) per 0.1 ml of liver homogenate after the livers were homogenized in 0.32 M sucrose solution (1 part liver, 3 parts homogenizing medium).

radioactivity has almost disappeared from the liver. The peak in RNA specific activity for deficient rats is also about 1 hour (Figure 3); however, the extent of incorporation is much less, at all times after uridine injection, than in the case of the normal animals. The disappearance of radioactivity from the deficient liver following ^{3}H-uridine injection, was faster than in case of normal liver.

Having obtained these results with deficient rats, vitamin A-normal rats were studied. It can be seen from Figure 4 that the rate of incorporation of labeled uridine into the total RNA of normal rat liver was also stimulated by the intraperitoneal injection of this large dose (10 mg) of potassium retinoate. On the other hand when lower dose (175 μg) of potassium retinoate was given by intraperitoneal injection into normal rats, this lower dose of potassium retinoate did not stimulate the rate of incorporation of labeled uridine into total RNA.

In the experiment reported in Figure 5 potassium retinoate was given by intraperitoneal injection to vitamin A-deficient animals that were then killed at various time intervals. Labeled uridine was administered by intraperitoneal injection 1.5 hours prior to killing in each case. As can be seen from the figure, maximum stimulation of incorporation of uridine into RNA had already occurred at 3.5 hours after potassium retinoate injection.

As a control experiment 10 mg of KCl was given by intraperitoneal injection to vitamin A-deficient rats. Labeled uridine was also intraperitoneally injected 1.5 hours before killing. The rats were killed at various time intervals following the KCl injection. As can be seen in Figure 5, KCl did not stimulate the rate of incorporation of

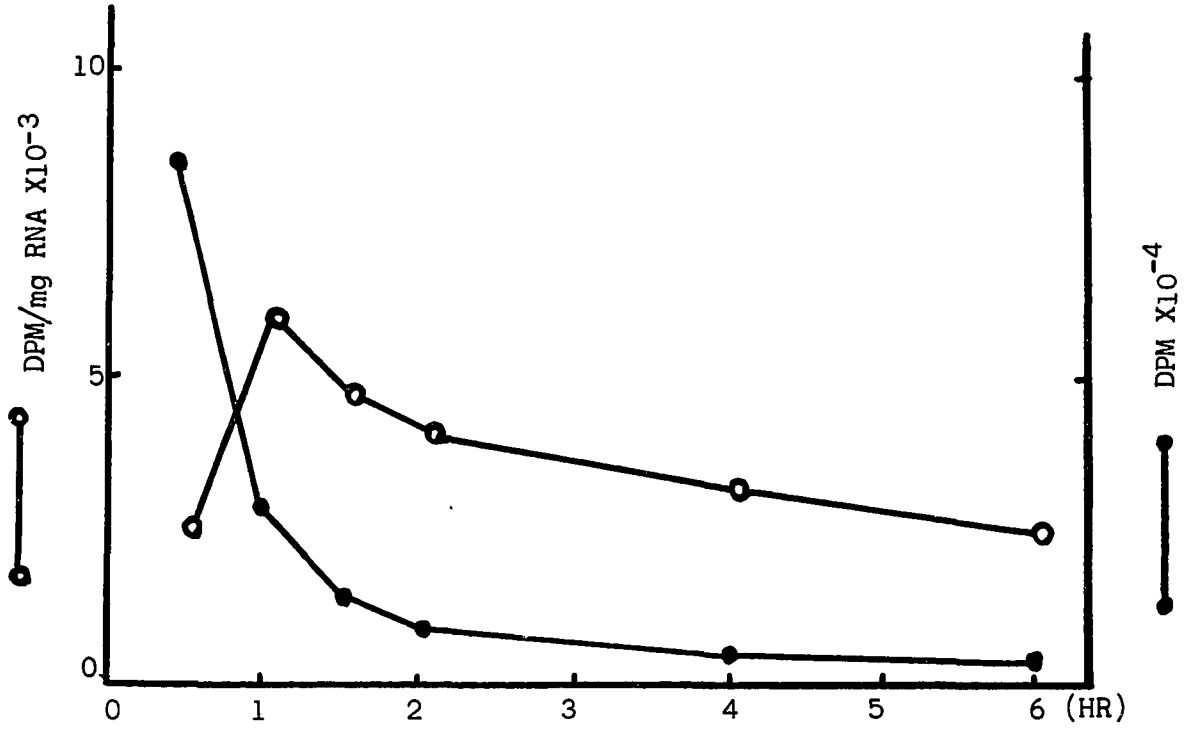


Figure 3. Effect of time from ^{3}H-uridine injection to sacrifice on rate of incorporation of ^{3}H-uridine into total RNA of deficient rat liver.

One hundred microcuries of ^{3}H-uridine were injected intraperitoneally into each rat at 0 time. Four rat livers were pooled to obtain each point.

O——O Radioactivity (DPM) per mg RNA.

●——● Radioactivity (DPM) per 0.1 ml of liver homogenate after the livers were homogenized in 0.32 M sucrose solution (1 part liver, 3 parts homogenizing medium).

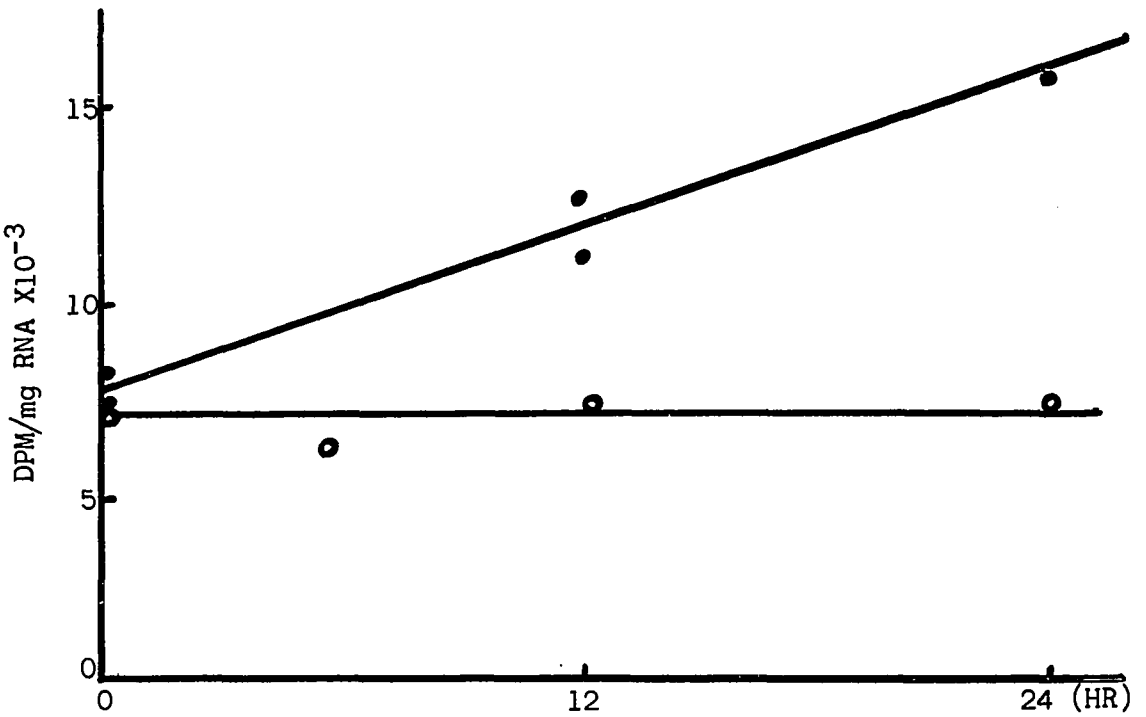


Figure 4. Effect of potassium retinoate injection on rate of incorporation of ^{3}H-uridine into total RNA of normal rat liver.

Ten microcuries of ^{3}H-uridine were injected intraperitoneally into each rat 1 hour before killing.

●——● Ten milligrams of potassium retinoate were given by intraperitoneal injection at 0 time. Two rat livers were pooled to obtain each point.

○——○ One hundred and seventy-five micrograms of potassium retinoate were given by intraperitoneal injection at 0 time. Four rat livers were pooled to obtain each spot.

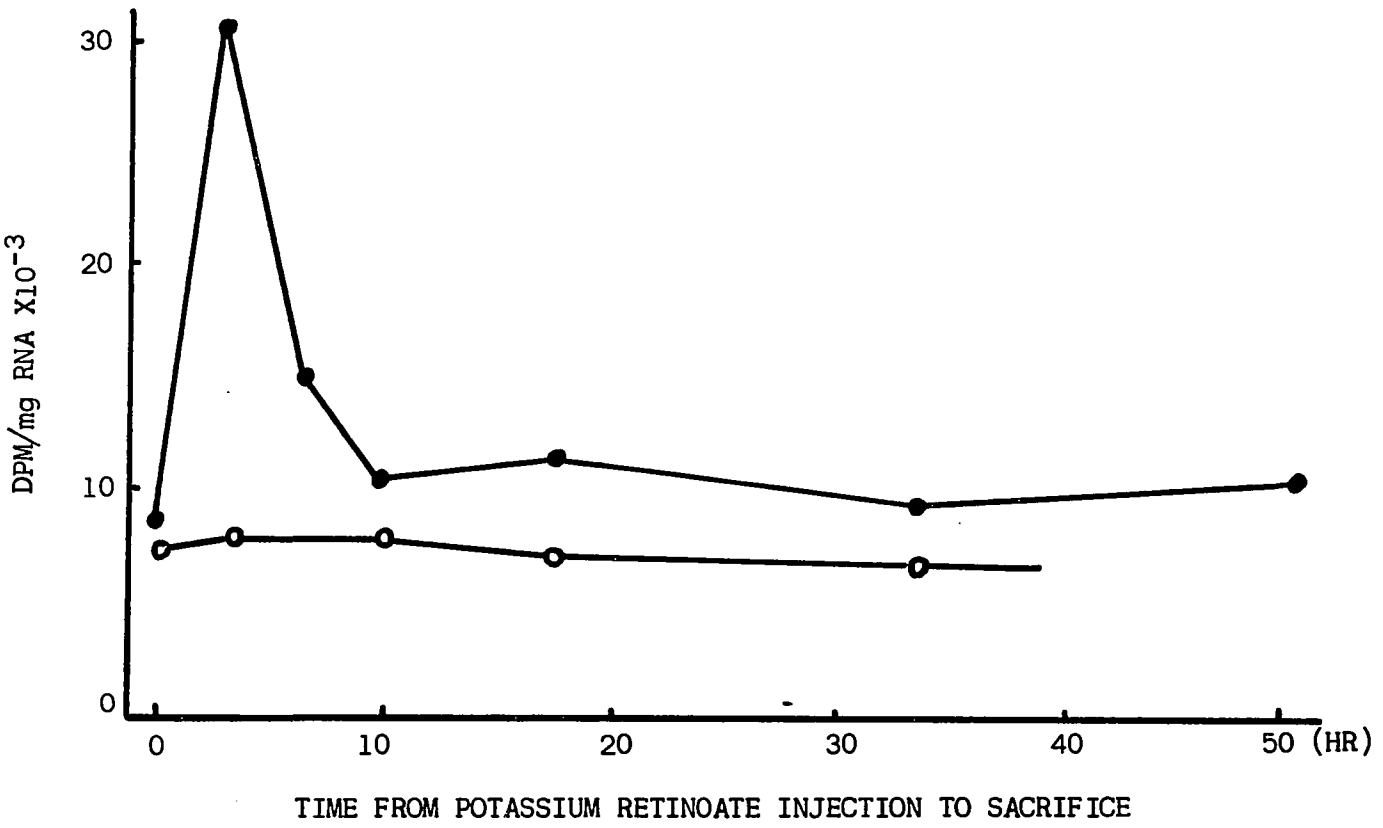


Figure 5. Effect of time from potassium retinoate injection on ^{3}H-uridine incorporation into total liver RNA.

Ten microcuries of ^{3}H-uridine were given by intraperitoneal injection 1.5 hours before killing. Each point represents four rats.

●——● Ten milligrams of potassium retinoate were given by intraperitoneal injection at 0 time.

o——o Ten milligrams of potassium chloride were given by intraperitoneal injection at 0 time.

labeled uridine into total liver RNA.

Although the cortisone administration experiment which will be described later in this chapter, was not designed as a control experiment, the results (shown in Figure 11) are useful as another control of above experiment (Figure 5).

Nuclear RNA

Following these experiments with total liver RNA, experiments were carried out with nuclear RNA fraction extracted from isolated rat liver nuclei at 65°C by 80% phenol. Potassium retinoate (0.35 mg) was given by intraperitoneal injection and animals were killed at varying time intervals thereafter. Labeled uridine was again given 1.5 hours before killing. Maximum incorporation into nuclear RNA was found to occur by 4 hours after potassium retinoate administration as shown in Figure 6.

A study was undertaken with regard to ^{3}H-uridine incorporation into nuclear RNA as a function of time after intraperitoneal uridine administration to deficient rats and to rats given 10 mg potassium retinoate 2 hours before killing. The data given in Table 2 are similar to those found for total liver RNA with no vitamin A administration as shown in Figures 2 and 3 and indicate that 1-1.5 hours is a satisfactory time to use to obtain good uridine incorporation. In the absence of vitamin A, little effect of time after uridine injection was observed at either 1 or 1.5 hours after uridine injection.

Since all the above experiments were carried out with ad libitum-fed rats an experiment was carried out using pair-fed normal, ad libitum-fed normal, and vitamin A-deficient animals. Pair feeding is a technique

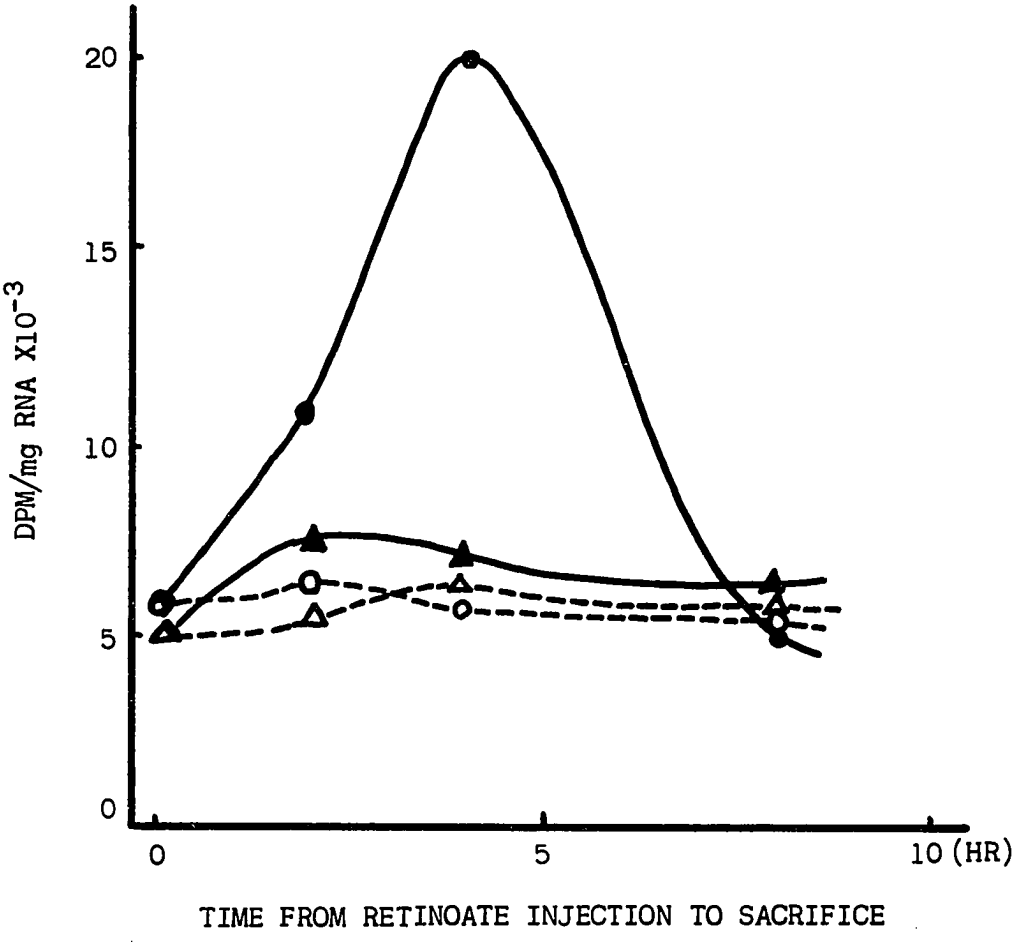


Figure 6. Effect of time from potassium retinoate injection to sacrifice on rate of incorporation of ^{3}H-uridine into RNA of deficient rats.

Potassium retinoate (0.35 mg) was given by intraperitoneal injection at 0 time. Five microcuries of ^{3}H-uridine were given by intraperitoneal injection 1.5 hours before killing. Each point represents four rats.

TABLE 2

EFFECT OF TIME, ON RATE OF ^{3}H-URIDINE INCORPORATION INTO LIVER NUCLEAR RNA FOLLOWING POTASSIUM RETINOATE (10 mg) ADMINISTRATION TO VITAMIN A-DEFICIENT RATS

Number of Rats	Time, Retinoate[a] to Killing	Time, ^{3}H-Uridine[b] to Killing	dpm/mg RNA
2	2 hr	0.5 hr	7.2×10^{3}
2	0 hr	0.5 hr	4.9×10^{3}
4	2 hr	1 hr	16.5×10^{3}
4	0 hr	1 hr	5.2×10^{3}
2	2 hr	1.5 hr	12.3×10^{3}
3	0 hr	1.5 hr	6.6×10^{3}

[a]Ten milligrams potassium retinoate given by intraperitoneal injection.

[b]Five microcuries ^{3}H-uridine given by intraperitoneal injection.

of regulating the food-intake of the normal animal by equating it with the amount of food eaten by the deficient animal. The goal was to eliminate variations in food-intake and inanition as factors in comparing vitamin A-deficient animals with normal animals. At daily intervals the quantity of food eaten by a deficient animal is measured and that amount of food is given to its normal pair-mate. In the beginning each animal is given an equal quantity of food. The data are given in Table 3. As can be seen from the data graphically presented in Figure 7, pair-feeding did not eliminate the effect of vitamin A status on uridine incorporation into rat liver nuclear RNA in the vitamin A-deficient pair-fed animals. In this experiment, the labeled uridine was given 1 hour before killing, and the results appear to indicate that the effect of the deficiency on uridine incorporation into liver nuclear RNA is more closely related to vitamin A status than to inanition.

A comparison of rates of incorporation of tritiated uridine into various liver RNA fractions of vitamin A-deficient rats, was carried out in order to be able to correlate the earlier total RNA data with the later nuclear RNA data. The results given in Table 4 show that while there are effects of the deficiency on all three fractions, the greatest effect was on the nuclear RNA fraction.

Since a nuclear RNA response to 0.35 mg of potassium retinoate was obtained, experiments were next undertaken using lower levels of potassium retinoate. Figure 8 reports the results for 1 hour uridine incorporation found at 3.5 hours after various levels of potassium retinoate, from 3 to 350 μg. In this experiment, times of 3.5 hours after potassium retincate and 1 hour after ^{3}H-uridine were arbitrarily chosen.

TABLE 3

COMPARISON OF ^{3}H-URIDINE INCORPORATION INTO LIVER NUCLEAR RNA IN VITAMIN A-DEFICIENT RATS, THEIR PAIR-FED CONTROLS, AND *AD LIBITUM*-FED CONTROLS

Group	Number of Rats	dpm/mg RNA
Deficient	5	4,360
Normal pair-fed	5	9,610
Normal *ad libitum*-fed	6	12,720

Five microcuries ^{3}H-uridine given by intraperitoneal injection 1 hr before killing. No food given after uridine injection.

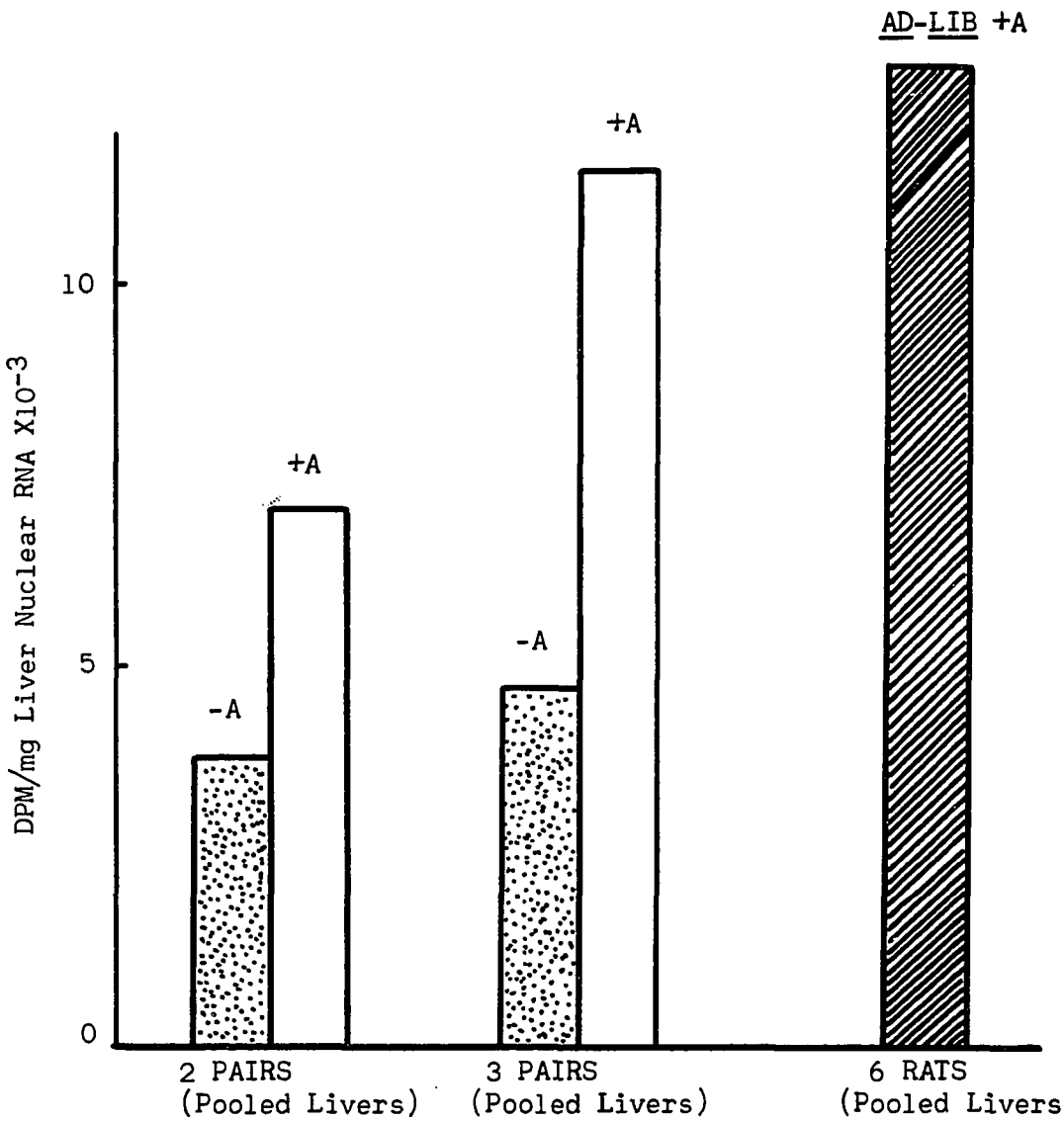


Figure 7. Comparison of rate of incorporation of ^{3}H-uridine into liver nuclear RNA of deficient rats (-A), pair-fed normal rats (+A), and ad libtum normal rats.

Five microcuries of ^{3}H-uridine were given by intraperitoneal injection 1 hour before killing.

TABLE 4

COMPARISON OF INCORPORATION OF ^{3}H-URIDINE INTO VARIOUS LIVER RNA FRACTIONS OF VITAMIN A-DEFICIENT AND PAIR-FED CONTROL RATS

Liver RNA Fraction	Number of Pairs of Rats	dpm/mg RNA	
		Vitamin A deficient	Pair-fed controls
Nuclear	2	3,500	7,300
	2	2,900	6,700
Cytoplasmic	2	12,400	15,000
	2	13,900	14,000
Total	2	10,100	14,800
	2	11,300	18,700

Five microcuries ^{3}H-uridine given by ip injection, 1-1/2 hr before killing.

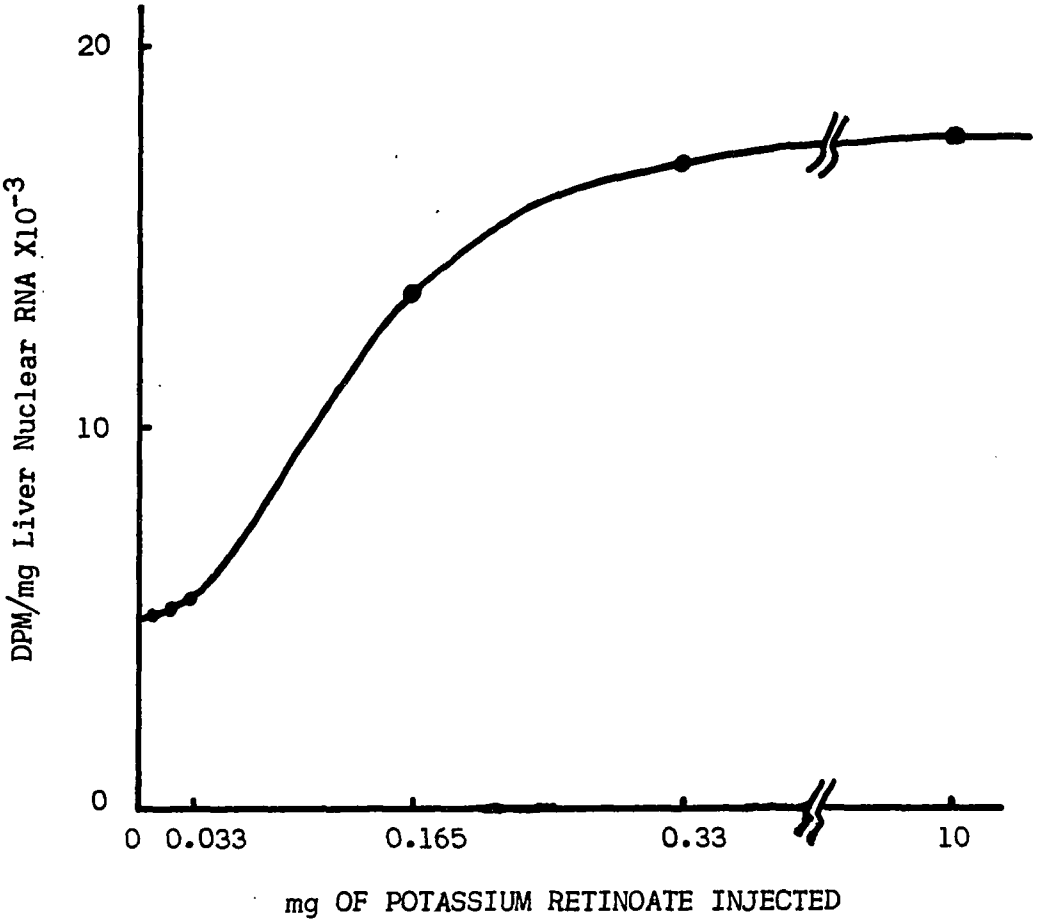


Figure 8. Effect of amount of potassium retinoate on rate of incorporation of ^{3}H-uridine into liver nuclear RNA of deficient rats.

Five microcuries of ^{3}H-uridine were given by intraperitoneal injection 1 hour before killing. Potassium retinoate was given intraperitoneally 3.5 hours before killing each case. Each point represents 5 to 6 rats.

shows at this 3.5 hour time interval a response to levels at least as low as 175 μg; however, no significant response was observed at 35 μg.

The data in Figure 8 were all taken at 3.5 hours after administration of vitamin A. It would be quite possible that a significantly longer time for an effect on liver nuclear RNA labeling would be required for an adequate amount of retinoate to reach the liver when such low levels are given intraperitoneally. This would vary with the amount injected intraperitoneally. Therefore, an experiment was undertaken to determine whether a much lower level of potassium retinoate, in this case 17 μg, would affect liver nuclear RNA labeling in deficient rats at any subsequent time.

Seventeen micrograms of potassium retinoate was administered by intraperitoneal injection and the animals were killed at various times thereafter and, as before, 1 hour after uridine administration. The data presented in Figure 9 show clearly a response in RNA labeling; however, at this low level of vitamin A administration the response is much slower, reaching maximum at about 16 hours instead of 4 hours. Presumably this is due to the time required to allow retinoic acid to be transported from the intraperitoneal injection site to the liver in adequate quantities to bring about this increased incorporation. Possibly with a very much lower amount administered it will take longer to build up liver levels, whereas very high levels administered will rapidly yield a high content in the liver.

Cortisone Administration

Because of earlier work on the effect of vitamin A deficiency on adrenal function and adrenal hormone formation (11) in which a "bio-

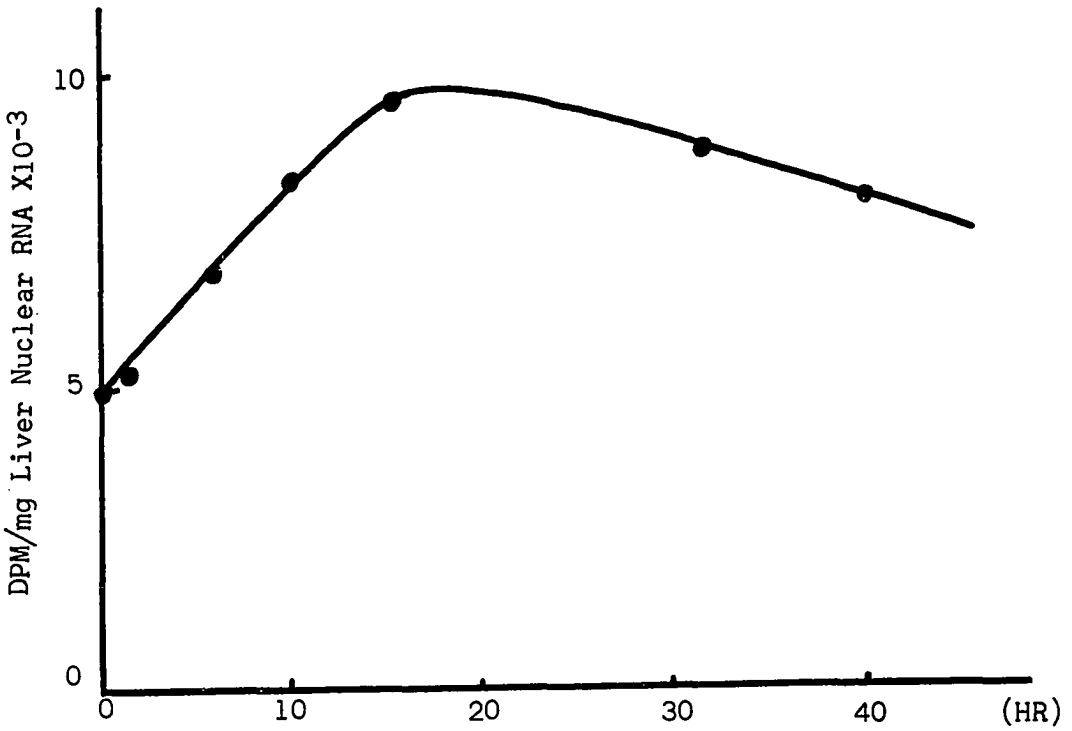


Figure 9. Effect of time after 17 μg potassium retinoate on rate of incorporation of ^{3}H-uridine into liver nuclear RNA of deficient rat.

Five microcuries of ^{3}H-uridine were given by intraperitoneal injection 1 hour before killing. Potassium retinoate (17 μg) was given by intraperitoneal injection at 0 time. Six rats were used per point.

chemical adrenalectomy" due to the deficiency was suggested, an experiment was next undertaken to study the effect of cortisone administration to vitamin A-deficient rats on rat liver nuclear RNA synthesis. The ^{3}H-uridine was given 1 hour prior to killing and animals were killed at time intervals of 1 to 5 hours after being given 5 mg hydrocortisone. As can be seen from Figure 10, hydrocortisone administration had no effect on uridine incorporation into liver nuclear RNA in these vitamin A-deficient rats.

Rapidly Labeled Nuclear RNA

Since the primary effect of vitamin A deficiency on RNA synthesis appeared to be in a nuclear fraction, which according to Lang and Sekeris (40) is high in messenger RNA activity, experiments were carried out to see whether rapidly labeled nuclear RNA was particularly affected by vitamin A. For these studies, ^{3}H-uridine was given by intravenous injection and the animals were killed 5 minutes later. The nuclear RNA was isolated as before. The data charted in Figure 11 show a four-fold difference in specific activity of this rapidly labeled nuclear RNA between vitamin A-normal and vitamin A-deficient animals. This difference can be visualized by expressing the RNA data in terms of disintegrations per minute per gram of liver (two-fold difference) or per milligram of RNA per gram of liver (two-fold difference).

Intestinal Mucosal RNA

Since intestinal mucosa is particularly sensitive to vitamin A deficiency, experiments were undertaken to study the effect of vitamin A deficiency on incorporation of labeled uridine into intestinal mucosal

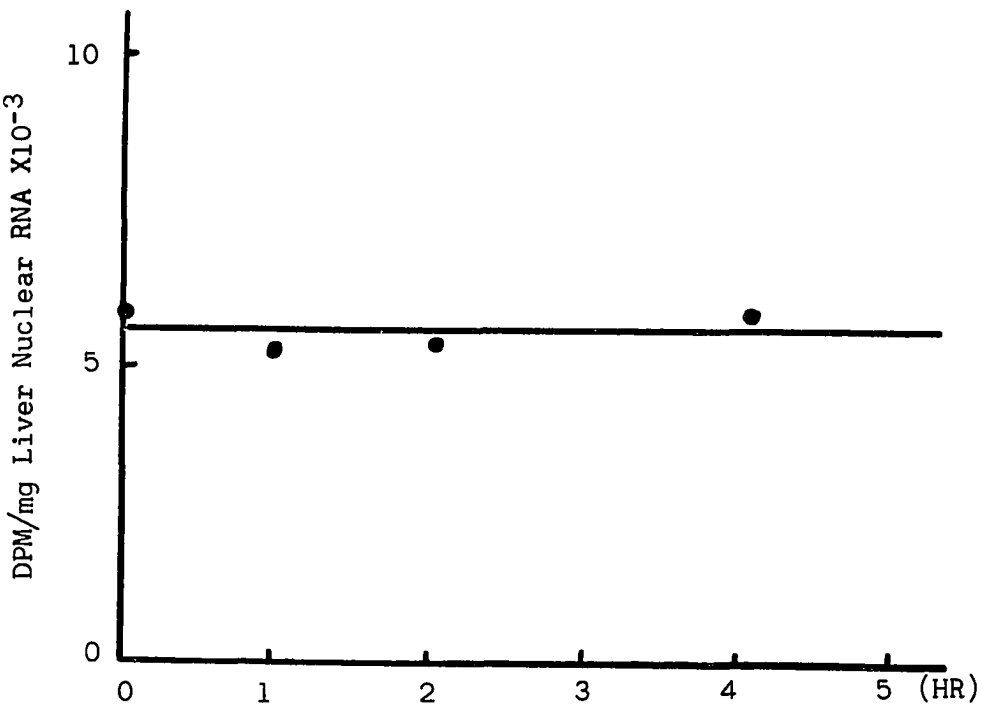


Figure 10. Effect of cortisone on rate of incorporation of ^{3}H-uridine into liver nuclear RNA of deficient rat.

Five microcuries of ^{3}H-uridine were given by intraperitoneal injection 1 hour before killing. Five milligrams of hydrocortisone acetate were given by intraperitoneal injection at 0 time. Two rats were pooled per point.

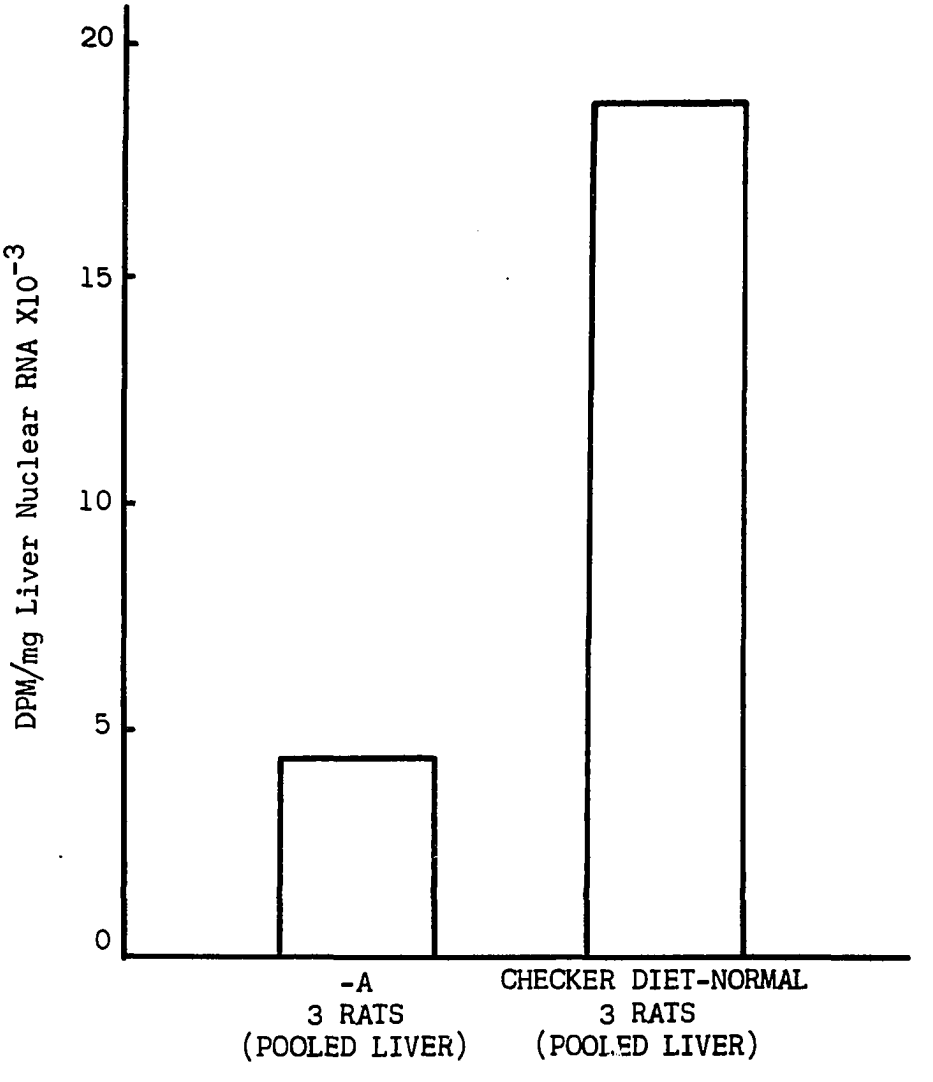


Figure 11. Comparison in amount of incorporation of ^{3}H-uridine into rapidly labeled liver nuclear RNA of vitamin A-deficient and normal rats.

Five microcuries of ^{3}H-uridine were given in 0.5 ml saline by tail vein. Rats were killed 5 minutes after tail vein injection.

RNA. Normal and deficient rats were given 5 μCi labeled uridine by intraperitoneal injection and animals were killed at various time intervals thereafter. Intestinal mucosal nuclear RNA was isolated and its radioactivity determined. As can be seen from Figure 12 there was at least a threefold difference in incorporation of labeled uridine into intestinal mucosal nuclear RNA between vitamin A-normal and vitamin A-deficient animals. Maximum incorporation appeared to occur about 0.5 hours after administration of labeled uridine for normal rats and 1 hour for deficient rats.

Having found an effect of vitamin A status on intestinal mucosal RNA synthesis and an effect on rapidly labeled liver nuclear RNA, experiments were undertaken in which rapidly labeled intestinal mucosal as well as liver RNA were studied concurrently. Vitamin A-deficient animals were treated with 0.175 mg potassium retinoate given by jugular vein injection. The animals were killed at various time intervals from 15 minutes to 15 hours after the potassium retinoate injections. The ^{3}H-uridine was also administered via the jugular vein 5 minutes before the animals were killed, following which both liver and intestinal mucosal nuclear RNA were isolated and counted. As can be seen from the data in Figure 13, the maximum response to potassium retinoate in both liver and mucosal rapidly labeled nuclear RNA occurred within the first 0.5 hour after administration of the vitamin.

This experiment was followed by a similar experiment in which 35 μg of potassium retinoate was given and the first time period was only 15 minutes. The vitamin was given by jugular vein injection and again 5 minutes before killing. Groups of rats were killed at various time in-

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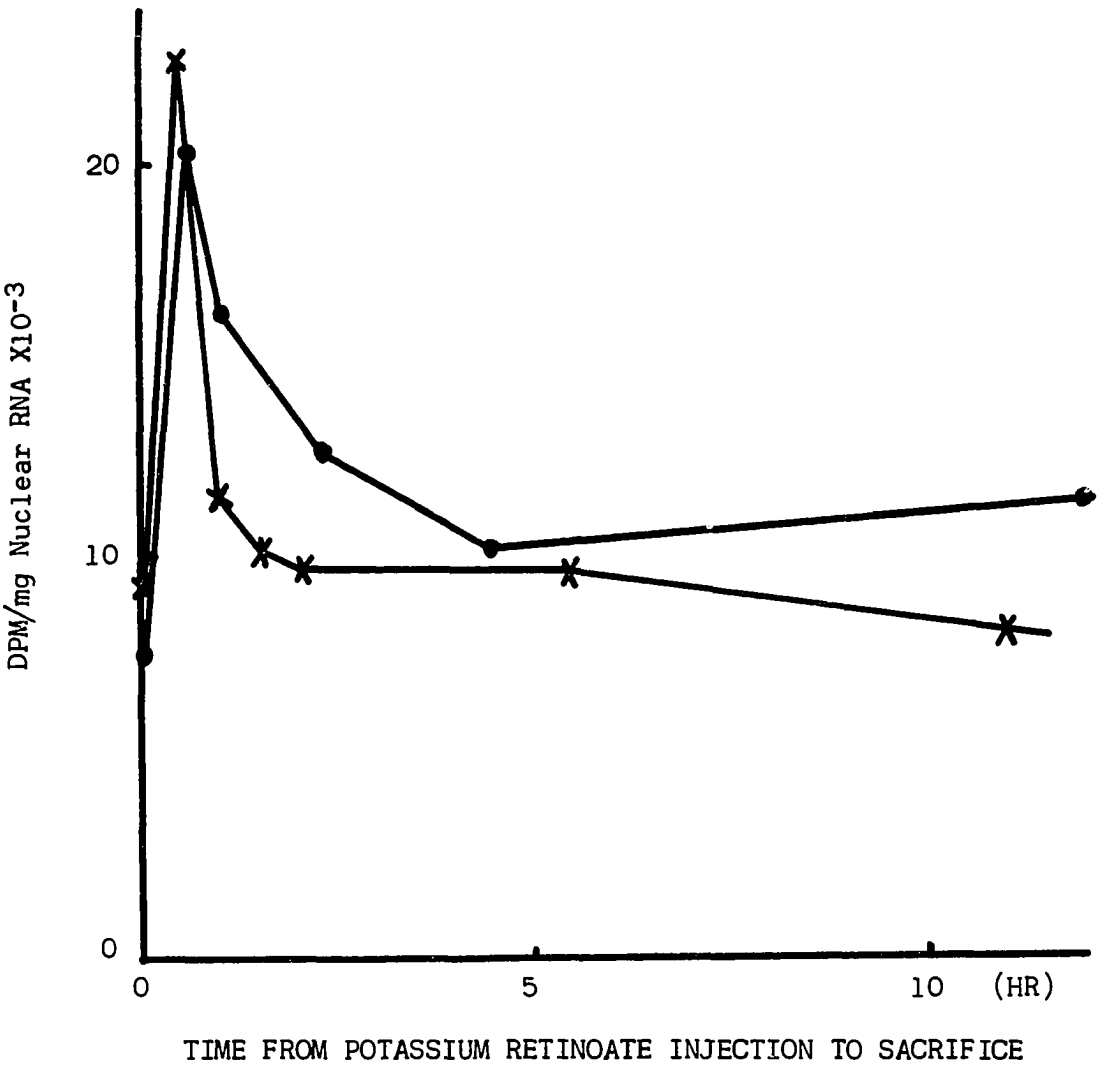


Figure 13. Effect of potassium retinoate on rate of incorporation of ^{3}H-uridine into rapidly labeled nuclear RNA of vitamin A-deficient rats.

Potassium retinoate (175 μg) was given directly into jugular vein at 0 time. Five microcuries of tritiated uridine were given directly into the same jugular vein 5 minutes before killing each animal. Data on intestinal mucosal RNA represent four rats per point. Data on liver nuclear RNA represent two rats per point.

●——● Liver
X——X Intestinal mucosa

tervals from 15 minutes to 14 hours after the single potassium retinoate injection. A control series of vitamin A-deficient rats were injected with 35 μg of KCl at zero time. As can be seen from Figure 14, the maximum labeling of the rapidly labeled RNA from the intestinal mucosa had occurred within 15 minutes after vitamin A administration. However, with this low "physiological level" of vitamin A there is a second slow increase in labeling from 4 to 14 hours. The liver RNA data are similar to the intestinal mucosal RNA data except that with this low vitamin A dose the initial peak in labeling is less pronounced and lower than in the previous experiment with a higher dose of vitamin A.

Sucrose Gradient Resolution

As mentioned above, the intravenous administration of potassium retinoate to vitamin A-deficient rats increased intestinal mucosal and liver nuclear RNA labeling. Based on the finding that liver nuclear RNA labeled in 5 minutes with ^{3}H-uridine was increased in labeling within 30 minutes after administration of 175 μg of potassium retinoate, the rapidly labeled liver nuclear RNA was fractionated on a 5 to 20% linear sucrose density gradient in order to investigate what portion(s) of the rapidly labeled nuclear RNA of vitamin A-deficient rat liver is stimulated by administration of potassium retinoate.

Total Liver RNA

Initially, the total RNA of normal rat liver and of vitamin A-deficient rat liver were fractionated on a linear sucrose gradient. The sedimentation profile indicated by solid lines and the radioactivity patterns indicated by broken lines are shown in Figure 15. The sedimenta-

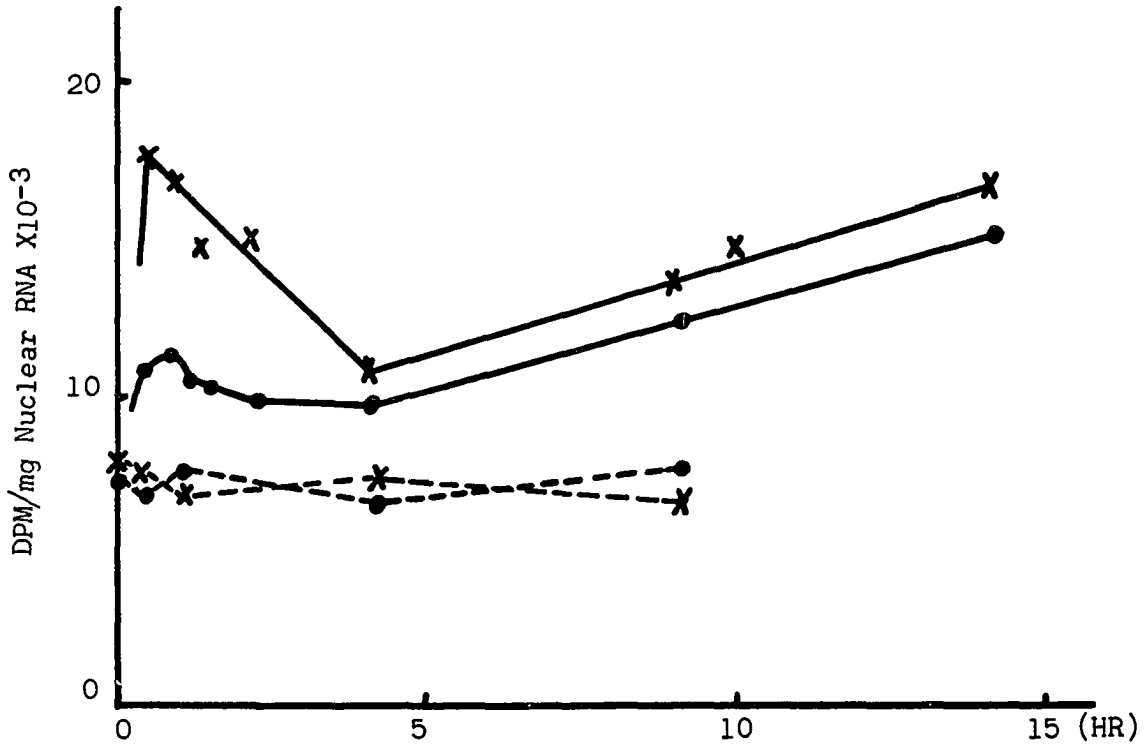


Figure 14. Effect of potassium retinoate on rate of incorporation of ^{3}H-uridine into rapidly labeled RNA of vitamin A-deficient rats.

(——) Potassium retinoate (35 μg) was given directly into a jugular vein at 0 time. Five microcuries of ^{3}H-uridine were given directly into the same vein 5 minutes before killing each animal. Data on the treated groups represent four rats per point. (●) Liver. (X) Intestinal mucosa.

(-----) Thirty micrograms of KCl were given by intrajugular vein injection at 0 time to the control rats. Data on the control groups represent two rats per point. (●) Liver. (X) Intestinal mucosa.

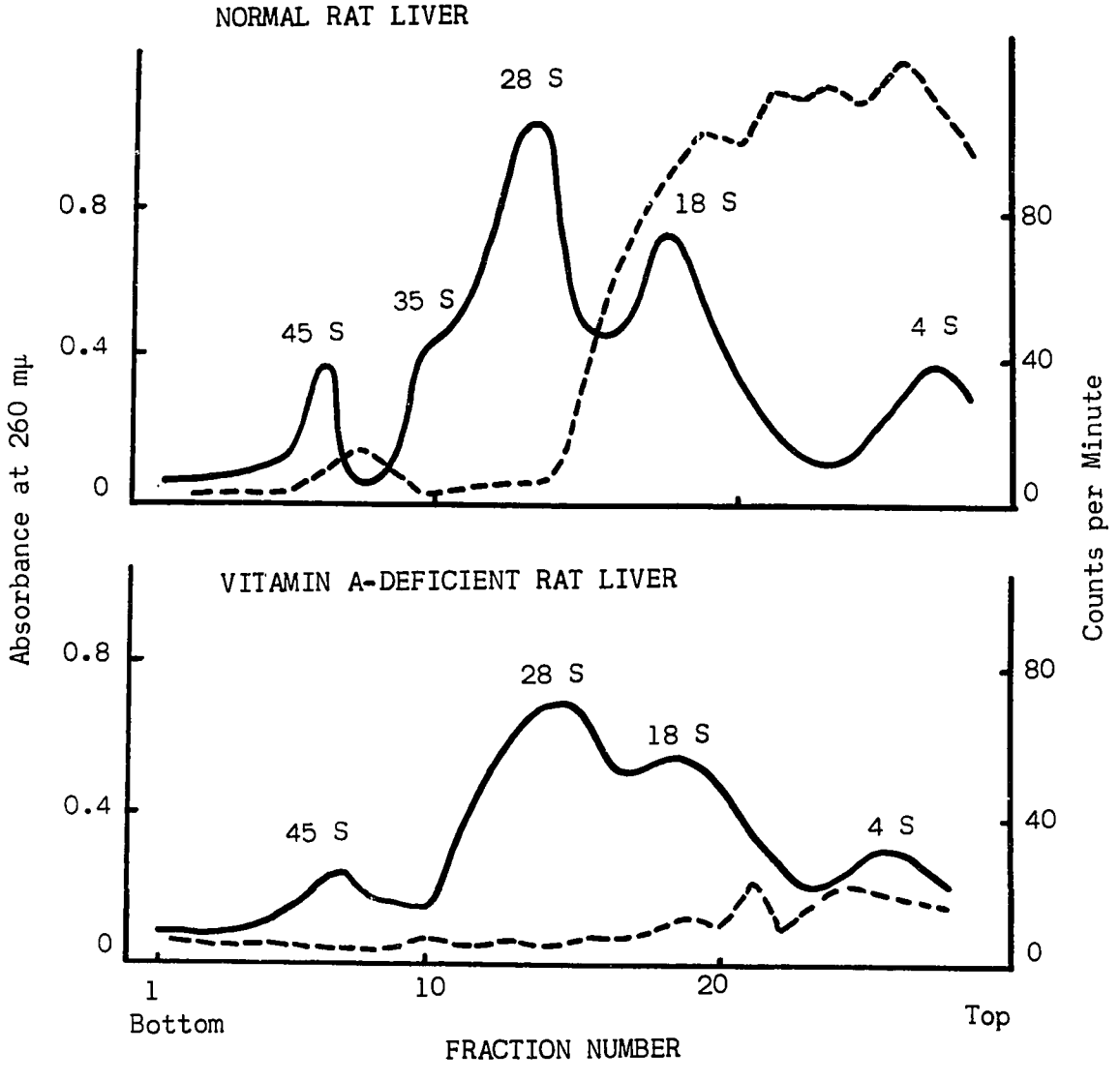


Figure 15. Sedimentation profiles of total RNA.

The details are given in the text. (——) Absorbance. (-----) Radioactivity.

tion profile is an absorbance curve at 260 mμ, the peaks of which indicate the fractions of the RNA solution fractionated. The sedimentation coefficients were taken from the data obtained by Roberts and Newman (48). The first fraction was taken from the bottom and the last fraction was taken from the top of the centrifuge tube. The peak of the radioactivity (counts per minute) indicates the rapidly labeled RNA fraction. As can be seen from Figure 15, rapidly labeled total RNA is mainly incorporated in fractions smaller than 28 S in both normal and deficient livers. However, the rate of incorporation into normal total RNA was much higher than into deficient total RNA. It has been found that the separation of 28 S from 18 S was poor in the sedimentation profile of vitamin A-deficient total RNA compared with that obtained using normal total RNA. This problem will be discussed in Chapter IV.

Nuclear Liver RNA

Figure 16 shows the comparison of the sedimentation profiles of nuclear RNA of normal rat liver with that of deficient rat liver. The sedimentation profile of normal nuclear RNA shown at the top of Figure 16 is similar to that of normal total RNA shown at the top of Figure 15, but a little different from that of deficient nuclear RNA shown at the bottom of Figure 16. In the sedimentation profile of vitamin A-deficient rat liver nuclear RNA, the height of the 28 S peak is much lower and almost the same as that of 18 S peak. The separation of 28 S from 18 S was slightly poorer than in case of liver nuclear RNA of vitamin A normal rats.

The rapidly labeled nuclear RNA of <u>normal</u> rat liver shown by the broken line at the top of Figure 16 is found in two fractions; the larger

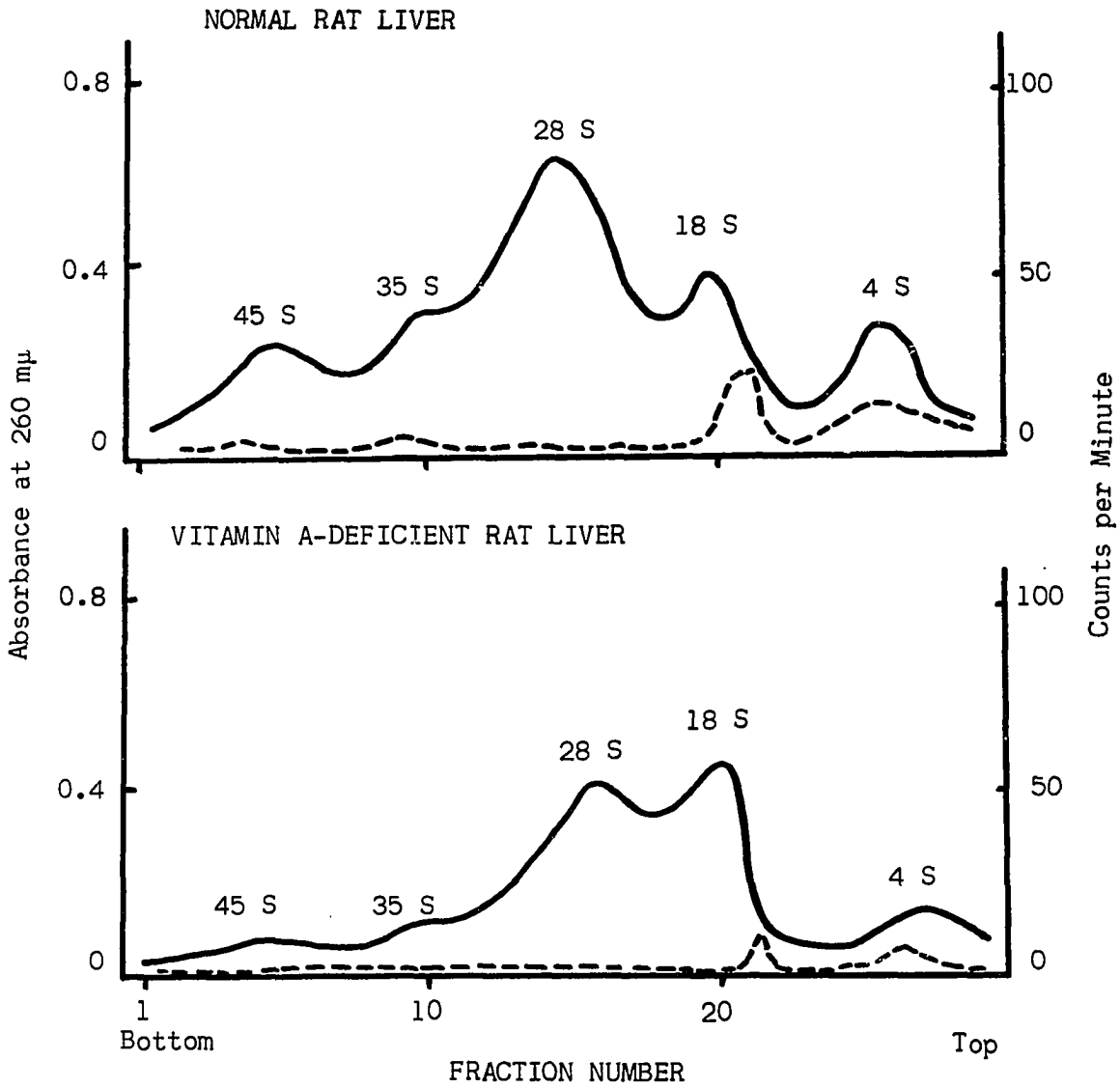


Figure 16. Sedimentation profiles of nuclear RNA.

The details are given in the text. (——) Absorbance. (-----) Radioactivity.

peak is just below the 18 S region and the smaller is near the 4 S region. The rapidly labeled nuclear RNA of deficient rat liver shown at the bottom of the figure is also found in apparently the same two fractions, but the amount of radioactivity incorporated in these peaks is much lower than in the case of the normal rat liver nuclear RNA. The results of both the overall sedimentation profiles and the uridine incorporation patterns indicate that vitamin A deficiency lowers the ability of synthesis of rapidly labeled nuclear RNA. If this is true, the administration of vitamin A to vitamin A-deficient rat must influence the synthesis of the rapidly labeled nuclear RNA of vitamin A-deficient rat.

In order to examine the effect of potassium retinoate administration on the synthesis of rapidly labeled nuclear RNA, 175 μg of potassium retinoate were given intravenously at various times before killing and 5 μCi of labeled uridine were given always 5 minutes before killing. Control experiments were carried out with the intravenous injection of 175 μg of potassium chloride to the animals 0, 20 or 40 minutes before killing. These three profiles at 0, 20 and 40 minutes were almost identical and are shown at the right hand bottom of Figure 17.

As can be seen from Figure 17, at 0 time treated with potassium retinoate, there were no significant absorbance peaks of RNA larger than 28 S. This seems to be characteristic of vitamin A deficiency (cf. Figure 16). However, approximately 2C minutes after the retinoate treatment, the 45 S species appeared and then the second peak, 35 S, appeared. By 90 minutes the sedimentation profile appears to be quite normal (cf. Figure 16).

Rapidly labeled nuclear RNA, indicated by the broken lines, ap-

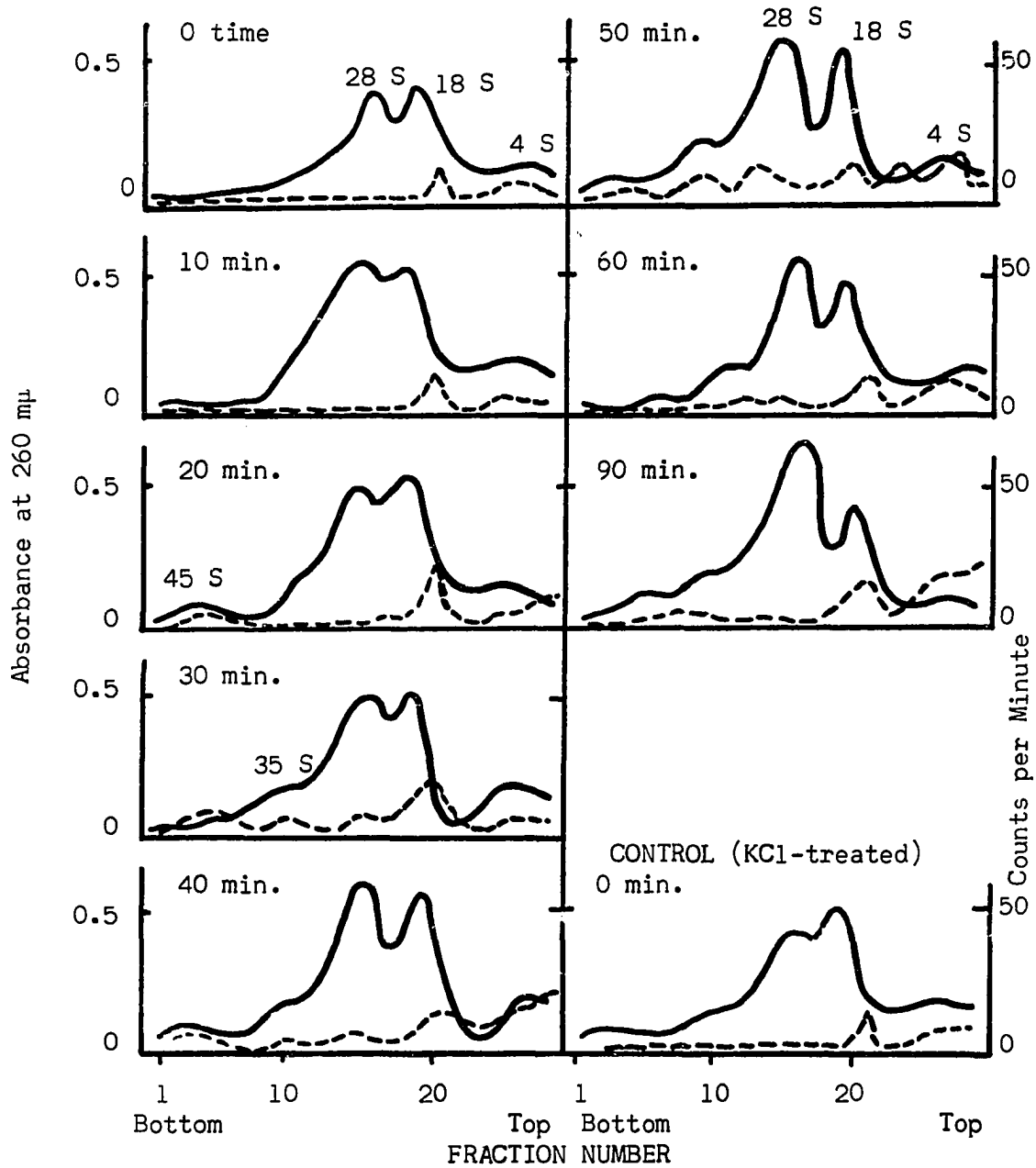


Figure 17. Sedimentation profiles of nuclear RNA of vitamin A-deficient rat liver treated with potassium retinoate.

The details are given in the text. (——) Absorbance. (-----) Radioactivity.

peared in the same species at 0 time as in normal nuclear RNA, i.e., below 18 S and near 4 S regions. However, the rate of incorporation of tritiated uridine into the RNA was much less than previously found in normal nuclear RNA and similar to that observed in deficient nuclear RNA (shown in Figure 16). However, potassium retinoate administration progressively charged this labeling pattern with time after potassium retinoate administration. This increase stimulating first the peak just below 18 S, reached a maximum by 20 minutes after potassium retinoate treatment, and then started to decrease gradually. As the labeling of this peak decreases the radioactivity in the 45 S and 4 S peaks increased. At 50 minutes the radioactivity of the 35 S peak was markedly increased. By 90 minutes the tritiated uridine incorporation pattern is close to that of the normal nuclear fraction shown in Figure 16. These changes in radioactivity shown by the broken lines are shown from the 0 time sedimentation profile to the 90 minutes sedimentation profile in Figure 17.

The patterns of the absorbance curve and the radioactivity curve obtained from the control experiments with KCl-treatment at 0, 20 and 40 minutes were identical to the 0 time treatment with potassium retinoate. The results of this experiment show that the injection stress of potassium chloride had no effect on rapidly labeled nuclear RNA synthesis in vitamin A-deficient rat liver.

Microsomal and Transfer Liver RNA

The next two figures (Figures 18 and 19) show the results of experiments designed to determine the possibility of effects on other RNA fractions. Figure 18 shows the profiles of three different species of microsomal RNA obtained from nutritionally different animals. The top

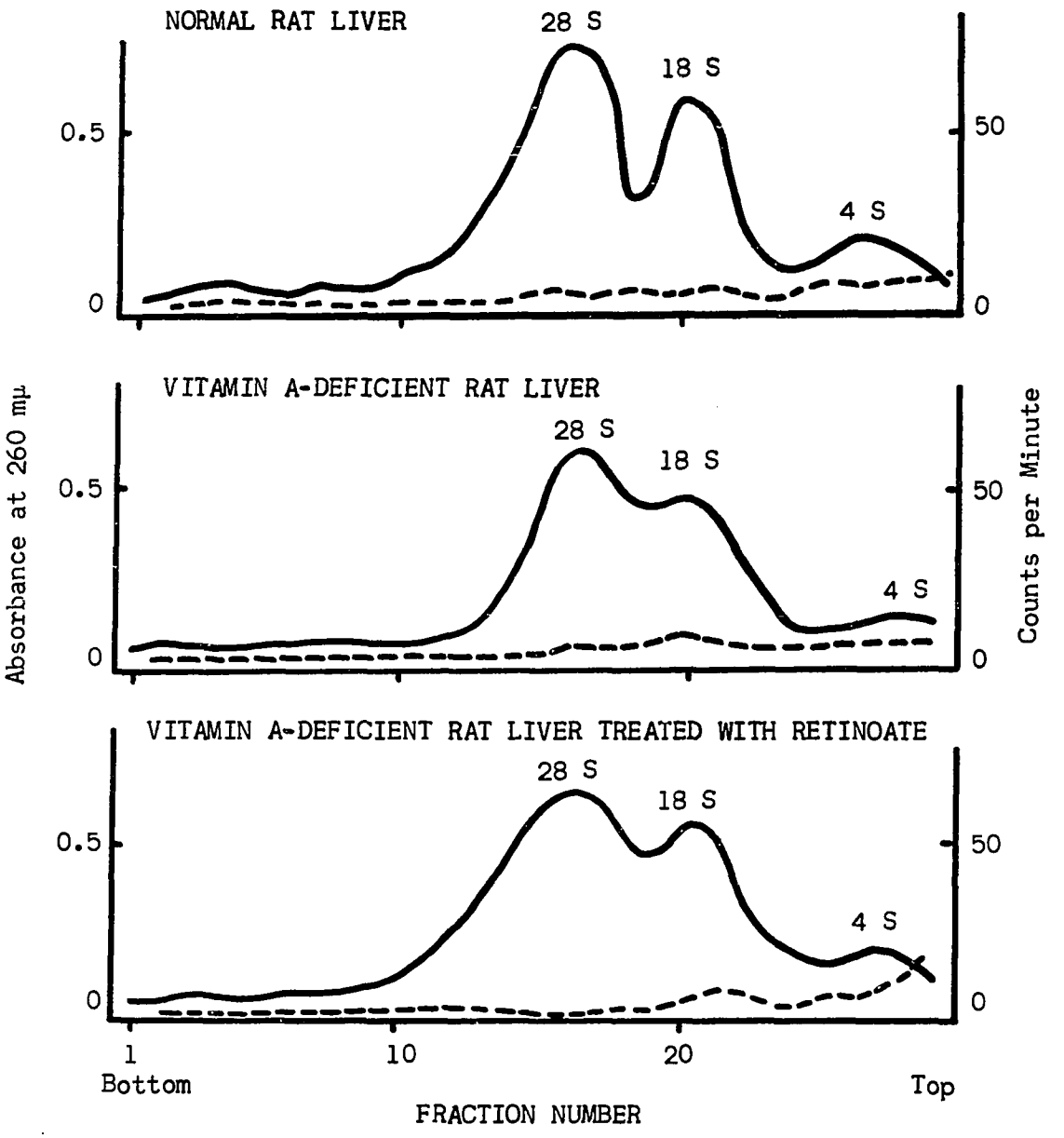


Figure 18. Sedimentation profiles of microsomal RNA.

The details are given in the text. (——) Absorbance. (-----) Radioactivity.

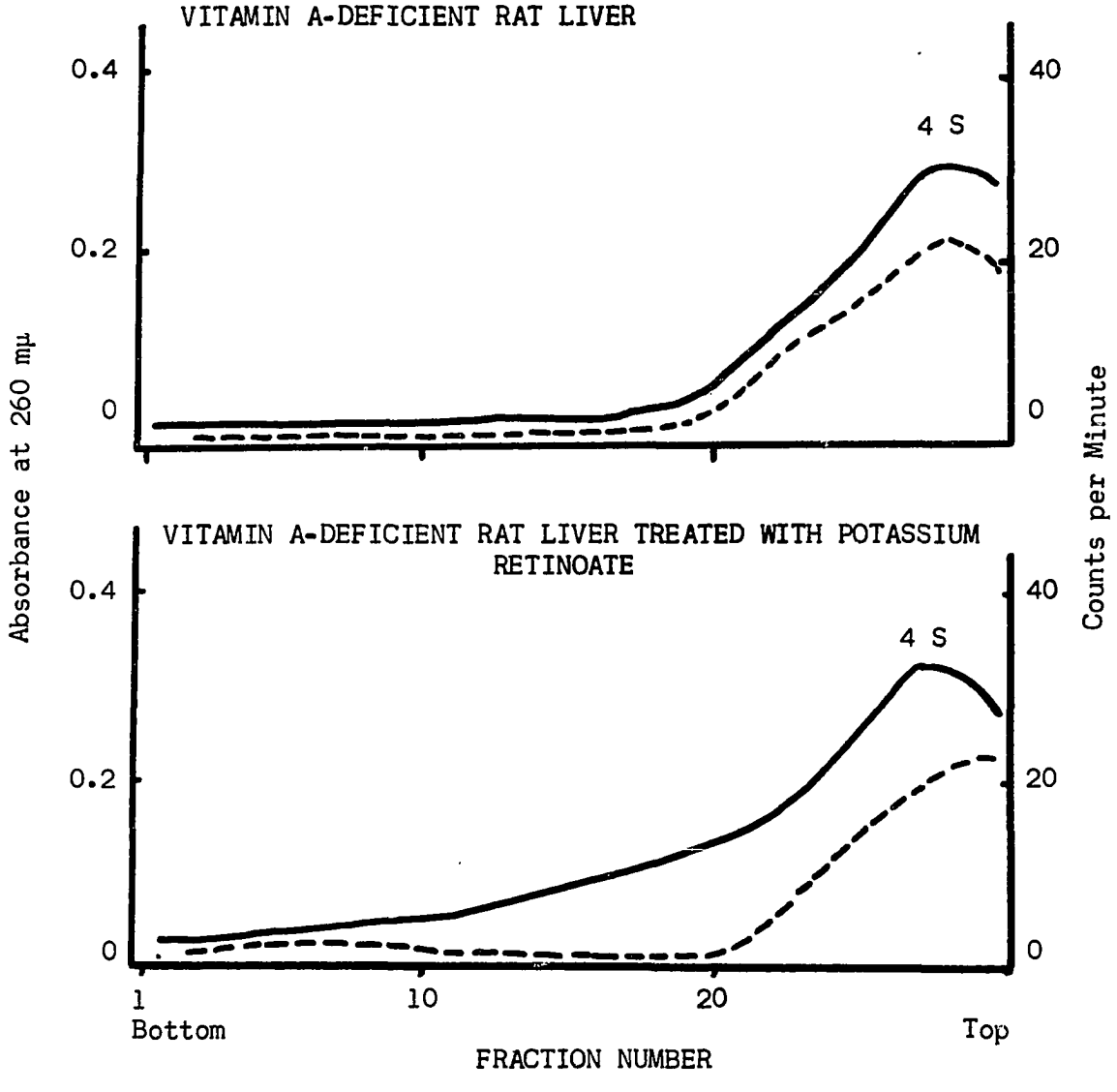


Figure 19. Sedimentation profiles of transfer RNA isolated from pH 5 fraction.

The details are given in the text. (——) Absorbance. (-----) Radioactivity.

is the profile of microsomal RNA obtained from normal rat liver; the middle one is the profile of microsomal RNA from deficient rat liver; and the bottom is from deficient rat liver treated with potassium retinoate. Almost identical patterns were obtained. This indicates that, under the experimental conditions employed, no significant amount of the radioactive uridine was incorporated into microsomal RNA, and potassium retinoate had no effect on the synthesis of microsomal RNA.

Figure 19 shows the profiles of transfer RNA isolated from the pH 5 fraction of vitamin A-deficient rat liver and of deficient rat liver treated with potassium retinoate 15 minutes before killing. Potassium reinoate showed no effect on transfer RNA synthesis in vitamin A-deficient rat liver.

Ribonuclease Digestion

Some of the nuclear RNA, microsomal RNA and transfer RNA solutions were digested by pancreatic ribonuclease prior to sedimentation. The samples treated with the enzyme were then subjected to ultracentrifugation under the same conditions described above. There were no peaks at 260 mμ; and the radioactivity was found at the top of the centrifuge tube. This indicates the destruction of the RNA samples by the ribonuclease.

CHAPTER IV

DISCUSSION

Introduction

From the data presented it seems clear that in some way vitamin A given as potassium retinoate rapidly increases the incorporation of labeled uridine into intestinal mucosal and liver nuclear RNA. This "turn on" of nuclear RNA synthesis by vitamin A occurs following the administration of physiological (35 μg) doses of potassium retinoate as well as after very high (10 mg) doses. With the lowest levels of potassium retinoate administration the first response appears to be much greater for intestinal mucosa than for liver nuclear RNA.

Injection Route of Potassium Retinoate

The time required for the "turn on" of nuclear RNA synthesis following vitamin A administration depends on the route of administration. Thus, the peak in stimulation of uridine incorporation appears at 3 to 4 hours following intraperitoneal injection of potassium retinoate and within 15 minutes when vitamin A is given intravenously.

When uridine incorporation into nuclear RNA is examined 1 or 1.5 hours after uridine administration the peak effect of potassium retinoate treatment of vitamin A deficient animals by intraperitoneal injection appears at about 4 hours after giving the vitamin. However, when very

rapidly labeled nuclear RNA is studied in animals killed 5 minutes after intravenous injection of uridine, the peak effect of potassium retinoate is found to occur very rapidly (within 20 minutes).

Potassium Retinoate Versus Retinol

A significant difference is noted in the response of RNA synthesis when injection of potassium retinoate versus injection of retinol are compared under the same experimental conditions (49). In the case of potassium retinoate a maximum response occurs at approximately 30 minutes. However, retinol injection causes a very slow increase up to 70 hours; no peaks appear. Thus, potassium retinoate is the more efficient form of vitamin A for the study of RNA synthesis.

Incorporation Rates of Normal and Deficient Animals

When normal and deficient animals are compared with regard to uridine incorporation into rapidly labeled RNA, a threefold difference is found. Also, an increased rate of synthesis is indicated, since the peak incorporation is reached at or before 30 minutes for the normal as compared to 1 hour in the deficient animals.

Vitamin A-DNA Relationship

The effect of potassium retinoate on the rate of incorporation of tritiated thymidine into DNA of vitamin A deficient rats has been examined (49). There is no significant effect on DNA synthesis up to 70 hours after the thymidine administration. The rate of incorporation begins to increase slowly in the retinoate treated rats after 70 hours. This increase may be due to the secondary effect of retinoate treatment.

Although vitamin A has no direct effect on DNA synthesis, vitamin A might have direct effects on transcribability of chromatin suggested by the report that chlorcquine which has a structure vaguely analogous to vitamin A, forms a complex with DNA (50), and by the report that dormin (abscisin), another possible vitamin A analog, exerts its leaf shedding effect by virtue of complexing with DNA and thus "shutting off" transcription (51, 52). Supporting the transcription site for vitamin A action is the observation by Raisz (53) that actinomycin D blocks the induction of bone resorption by vitamin A. The finding of De Luca et al. (10) that certain aminoacyl-t-RNA synthetases of the intestinal mucosa are decreased by vitamin A deficiency may be the mechanism whereby the effect of vitamin A on transcription is exerted on protein synthesis.

The effect of high levels of vitamin A on the production of almost 100% incidence of birth defects could be well explainable on the basis of an effect of vitamin A on DNA transcription (54, 55, 56).

Hormonal Effect on Rapidly Labeled Nuclear RNA Synthesis

Tata (57) and Tata and Williams-Ashman (58) have shown cell membrane changes brought about by various hormones. However, in these cases, the increase in RNA synthesis reached a maximum in about 24 hours (59). On the other hand, stimulation of rapidly labeled nuclear RNA in the uterus by estradiol-17 β is one of the earliest (2 to 20 minutes) responses of that organ (60, 61, 62), and they concluded (63) that estrogen regulates the rate and amount of genetic translation in the uterine cytoplasm by differentially controlling genetic transcription. An interesting observation has been reported (64) that cyclic-3',5'-adenylic acid

stimulates uridine incorporation in the uterus, taken from the ovariectomized rat and incubated for 1 hour in the presence of the nucleotide. It has also been reported that the generation of cyclic AMP occurs 15 seconds after the rat uterus is stimulated by estrogen (65), although its significance is yet uncertain in this case. Interestingly, vitamin A-deficient female rats do not respond to estrogen (66), indicating that the site of action of vitamin A may be metabolically before the site of action of estrogen.

Sucrose Density Resolution

The results from sucrose density analysis of the RNA samples isolated as described above, show that administration of potassium retinoate to vitamin A-deficient rats quickly stimulates the synthesis of a particular portion of rapidly labeled nuclear RNA, but does not significantly stimulate the synthesis of rapidly labeled microsomal and transfer RNA. As can be seen from Figure 17, potassium retinoate stimulated the synthesis of a particular portion of rapidly labeled nuclear RNA of vitamin A-deficient rat liver (shown by the broken lines) which was found just below 18 S region within 20 minutes. However, incorporation into this peak stimulated by potassium retinoate administration decreased 30 minutes after potassium retinoate administration, along with an increase in incorporation into the 45 S and 4 S peaks. At 50 minutes increases in incorporation into all heavier RNA fractions were found as well.

The sedimentation profile (shown by a solid line) and radioactivity curve (shown by a broken line) of nuclear RNA of vitamin A-deficient rat liver treated with potassium retinoate 90 minutes before killing (also shown in Figure 17) are almost identical to that of nuclear

RNA of normal rat liver. Although it takes more than a week to cure the symptoms of vitamin A deficiency of vitamin A-deficient rat after giving potassium retinoate, potassium retinoate administration to vitamin A-deficient rat quickly affects the RNA synthesis at the molecular level.

It seems that a particular portion of rapidly labeled nuclear RNA (below 18 S) is the precursor(s) of some other RNA fraction. Drews and Brawerman (67) reported that in rapidly labeled RNA from rat liver, the largest amount of template activity was found in the 9-16 S sedimentation zone. Rousseau and Crabbe (68) also found that rapidly labeled RNA in toad bladder was located in portions smaller than 18 S but larger than 4-6 S. Morimoto et al. (69) also reported that rapidly labeled nuclear RNA from calf thymus was found in the 9 S region.

Since, when 175 μg of potassium retinoate is intravenously given to a normal rat 15 minutes before killing, no appreciable change in both the sedimentation patterns and the rate of incorporation of tritiated uridine into rapidly labeled liver nuclear RNA has been observed compared with the case without any retinoate treatment, the retinoate effectively stimulates the synthesis of rapidly labeled nuclear RNA of vitamin A-deficient rats under the conditions employed. However, if potassium retinoate is given to normal rats long enough before killing it also stimulates RNA synthesis (shown in Figure 4).

Another interesting finding in this sucrose density analysis is a characteristic sedimentation pattern of vitamin A-deficient RNA. One of the serious technical difficulties involved in obtaining the sedimentation profile of vitamin A-deficient RNA was poor separation of peaks of the 18 S to 28 S fractions. Several possible combinations of time and

speed of the ultracentrifugation, density range of the sucrose solution, and the buffer for the sucrose solution, have been examined to obtain better separation of the 18 S to 28 S peaks. However, all attempts were unsuccessful in deficient RNA while reasonable separation of the normal RNA profile and of the deficient RNA profile treated with potassium retinoate before killing, were obtained. This poor separation of the 18 S to 28 S fractions appears to be a characteristic of the vitamin A-deficient RNA profile. Vitamin A may be involved, somehow, in differentiation of 18 S and 28 S RNA in the cells, i.e., in packaging or processing of nuclear RNA.

From the data obtained from this investigation, it is difficult to conclude the exact functional site of vitamin A in the animal body. However, it demonstrates that the administration of retinoic acid stimulates the synthesis of a specific rapidly labeled liver nuclear RNA (below 18 S) within 10 to 20 minutes. Furthermore, this rapidly labeled nuclear RNA (below 18 S) could act as a precursor of the 45 S RNA fraction, which may be responsible for the synthesis of a certain particular protein, the lack of which would cause the symptoms of vitamin A deficiency in the animal body.

There is an alternative hypothesis based on an observation that the synthesis of the 4 S fraction is increased, following the decrease of the rapidly labeled RNA fraction (below 18 S). This hypothesis is that the rapidly labeled nuclear RNA fraction (below 18 S) may be a precursor of 4 S fraction and be dissociated to the 4 S fraction at a later time.

Another possibility is that the rapidly labeled RNA fraction

(below 18 S) is a precursor of both 45 S and 4 S fractions.

All of these three possible ways to interpret the data obtained from this investigation will be studied in the future. The effect of vitamin A deficiency and vitamin A therapy on pool sizes and turn-over rates of uridine, uridine phosphates and RNA must be investigated although the apparent specificity of the vitamin A effect argues against a "dilution by pool" explanation.

Another problem arising from the data obtained in this investigation is the possibility of a toxic effect of vitamin A on RNA synthesis. It has been reported that when a large dose ($2\text{-}5 \times 10^6$ International Units) of vitamin A or its palmitate was given by mouth to rabbits it stimulated labeled amino acids incorporation into the plasma protein due to the toxic effect (70). However, when the total amount of vitamin A palmitate administered was less than 2×10^6 units, changes in acute-phase protein were usually negligible. The amount (175 μg = 500 units) of potassium retinoate used in this investigation appears to be low to cause toxicity. It will be studied in the future.

At present, no proof can be given as to whether the observed effect of vitamin A administration on RNA synthesis is direct or indirect, nor can light be shed at the moment on a mechanism for the effect.

CHAPTER V

SUMMARY

(1) The administration of retinoic acid, a biologically active form of retinol, in the form of the potassium salt, to vitamin A deficient rats stimulates the synthesis of rapidly labeled nuclear RNA in both intestinal mucosa and liver. The rapidly labeled nuclear RNA, in liver, stimulated by potassium retinoate administration within 15 minutes, is found below 18 S region on linear sucrose density gradient. This particular rapidly labeled nuclear RNA fraction may be precursor of another RNA, possibly the 45 S RNA fraction. The administration of the retinoate also stimulates, directly or indirectly, the synthesis of the 4 S rapidly labeled nuclear RNA fraction. In the former case, the administration of the retinoate may be involved in messenger RNA synthesis in the nucleus; in the latter case it may be involved in transfer RNA synthesis. If this interpretation is correct, the administration of potassium retinoate may regulate protein synthesis at the transcriptional level.

(2) Potassium retinoate effects RNA synthesis more markedly in the intestinal mucosa than in the liver, but is not effective in the kidney.

(3) Retinol has less effect than potassium retinoate on the

synthesis of rapidly labeled nuclear RNA in both liver and intestinal mucosa.

(4) The administration of potassium retinoate does not significantly affect DNA synthesis in either intestinal mucosa or in liver of normal and vitamin A-deficient rats.

(5) The possible functional routes of vitamin A are summarized in Figure 20.

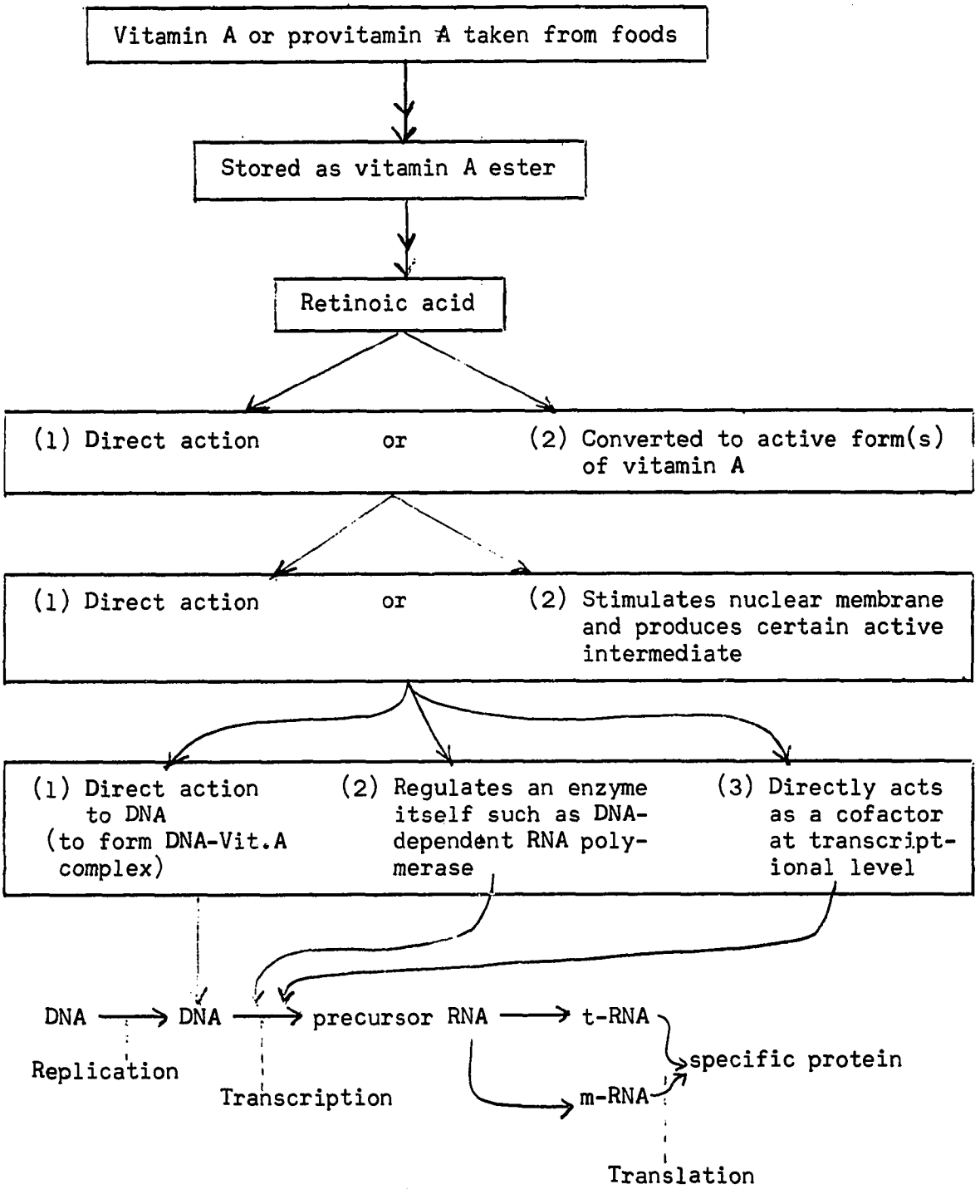


Figure 20. Possible functional routes of vitamin A.

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