THE EFFECT OF VITAMIN A ON PLASMA GLYCOPROTEINS

by

TIMOTHY CHARLES KIORPES

Submitted in Partial Fulfillment

of the Requirements for the

Degree of Master of Science

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 1, 1973

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ABSTRACT

The rate of uptake of label from radioactive D-glucosamine and D-mannose into the plasma glycoproteins was studied in vitamin A-deficient rats by comparison with the plasma of normal pair-fed controls. Preliminary studies indicated that peak incorporation (specific activity) was reached three hours after intraperitoneal injection with labelled sugar in both vitamin A-deficient and normal rat plasma. Normal-deficient pairs were injected with the same sugar, labelled with a different isotope, and their plasma was mixed based on equal amounts of protein and fractionated on DEAE-Sephadex A-50. There was a consistent decrease in radioactivity observed in what appeared to be the $alpha_1$ peak in vitamin A-deficiency. This depression was on the order of 30%, when normal and deficient peak totals were compared. This effect appeared with mannose and glucosamine and was of equal magnitude for both sugars. Fractionation of this peak by gel filtration showed that most of the radioactivity was associated with one glycoprotein, which was homogeneous in 5% polyacrylamide gel electrophoresis; the molecular weight of this glycoprotein was estimated to be on the order of 1×10^6 from its behavior on Sepharose 6B. The decrease in the incorporation of label into this peak was interpretted as representing a decreased synthesis rate in vitamin A-deficiency.

A shift in the position of the peaks occurred on DEAE-Sephadex in two fractionations of glucosamine-labelled plasma. The vitamin A-deficient plasma glycoproteins were eluted slightly later than those from normal plasma, indicating either a higher negativity in deficiency or a lower molecular weight. This effect was not investigated. However, its failure to be expressed during gel filtration and its reappearance in electrophoresis suggested that charge differences were responsible for this shift.

Thesis Supervisor: Dr. George Wolf
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ACKNOWLEDGEMENTS

This has not been an easy task to complete, but it would have been truely herculean without the moral support supplied by many people.

First, I am deeply indebted to Dr. George Wolf, whose patience has, no doubt, been well tried at times over the past four years. His encouragement has been instrumental in the development and completion of this thesis.

In the lab, I must thank Norah Butler, not only for her role in directing me towards relevent publications, but for tolerating my invasion into territory which could have been incorportated into her own thesis. In general, everyone in the lab has contributed something. And whether it was a can of Coke or a kick in the pants, it all seems to have helped.

Many thanks go to the crowd in Natick for cooking my meals for all these months, for waking me up in the morning and for putting up with the somewhat odd life-style thesis writing has necessitated.

Lastly, I tip my tired hat to the staff at *The Tech*, who have allowed this last piece of "graft and corruption" to be had. Thanks.

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LITERATURE REVIEW

A. Vitamin A

Vitamin A (retinol) is a fat-soluble, polyisoprenol. Countless studies, undertaken since its discovery (McCollum and Davis, 1913), have linked vitamin A to the maintenance of vision and of reproductive function, to the formation of bone tissue and to epithelial cell differentiation (De Luca and Wolf, 1969). While the physiological effects in vitamin A-deficient animals have been well documented, the precise molecular function of the vitamin has only been established in vision, where vitamin A aldehyde (retinal), bound to the protein opsin, functions as a photoreceptor in scotoptic vision (Wald, 1953). It is doubtful that the participation of the vitamin in functions other than vision parallels that found in the eye.

Evidence published by Varandani, *et al.* (1960) suggested a coenzymatic function for vitamin A in the synthesis of 3'phosphoadenosine-5'-phospho-sulfate. Subsequent work by many authors, showing a decrease in the activity of the enzyme ATP—sulfurylase, and other enzymes as well, in tissues from vitamin A-deficient animals, failed to establish this coenzymatic role (Rogers, 1969). The findings of Levi, *et al.* (1968), from their work with ATP-sulfurylase, indicated that the lower activities of these enzymes resulted from protein deficiency, which is a secondary effect of vitamin A-deficiency.

The initial observation by Wolbach and Howe (1925) of the degeneration of epithelial mucus-secreting cells and their keratinization in vitamin A-deficiency has led to investigations of these tissues. Fell (1963) found that the presence or absence of vitamin A determined whether stem cells would differentiate into mucus-secreting or keratinized epithelia in chick

embryo explant in culture. The principal constituent of mucus is glycoprotein. Realizing this fact, De Luca, *et al.* (1969) investigated glycoprotein synthesis in epithelial tissues. In rat intestinal mucosa, they measured amino acid uptake from labelled precursors into protein synthesized on the membrane-bound polysomes and found it to be depressed in vitamin A-deficiency. The synthesis of a specific fucose-containing glycopeptide decreased 50-70% in vitamin A-deficiency as shown by parallel incubations carried out *in vitro* using the intestinal mucosa of pair-fed normal and deficient rats (De Luca, *et al.*, 1970). This effect was demonstrated to occur *in vivo* as well. The investigation of endogenous levels of various transfer-RNAs showed these to be reduced in the intestinal mucosa of deficient rats (De Luca, *et al.*, 1971). This reduction resulted in a decrease in amino acid receptor activity, and hence protein synthesis. Similar decreases in glycoprotein biosynthesis have been noted in corneal epithelium and lung as well (De Luca and Wolf, 1972).

Related to the findings in intestine, Prutkin (1968) reported that vitamin A increases protein (presumably glycoprotein) synthesis in skin tumors (keratoacanthomas). Levinson and Wolf (1972) found the synthesis of a fucose-containing glycopeptide to increase in rabbit ear tumors after treatment with retinoic acid. Along with the cumulative results of De Luca and his co-workers, these studies indicate a relationship between vitamin A and the synthesis of glycoproteins in epithelial tissue. However, the level and the exact nature of this interaction remains unclear.

Vitamin A has been linked to the maintenance of membrane stability in the rat (Roels, *et al.*, 1969), specifically those of the liver lysosomes and of the erythrocyte membranes. In this capacity, retinol would seem to play a passive role in tissue with the exception of a possible effect on the binding

of ATPase by membranes. However the existence of retinol in the lysosomal membrane has recently been brought into question by Nyquist, *et al.* (1971), who in determining the distribution of the vitamin in liver cells, did not detect any associated with the lysosomal or mitochondrial membranes. The Golgi apparatus contained the greatest concentration.

An active function for membrane-associated vitamin A is suggested by the work of De Luca, *et al.* (1970). From incubations with ^{3}H-retinol and ^{14}C labelled GDP-mannose with a rat-liver microsomal preparation, they were able to isolate a mannose-lipid compound, labelled with both ^{3}H and ^{14}C. In microsomal fractions from vitamin A-deficient rats, the formation of this "manno-lipid" was stimulated by retinol. Labelling studies using ATP-^{32}P revealed that this "manno-lipid" contained a mono-phosphate linkage; acid hydrolysis released mannose and a metabolite of retinol from this compound (De Luca, *et al.*, 1973). *In vivo* formation was demonstrated as well as the specificity of this system for D-hexoses. The synthesis of a retinylphosphate-galactose compound has also been recently reported in a mast cell tumor (Helting and Peterson, 1972).

It has been proposed (Tetas, *et al.*, 1970) that a polyprenol lipid carrier may be involved in the transport of sugar moieties across the membranes of the endoplasmic reticulum similar to that implicated in the biosynthesis of the bacterial cell wall. Such a hypothesis is necessary to account for the movement of nucleotide sugars from the cytoplasm, where they are formed, into the channels of the endoplasmic reticulum, where the glycosyltransferases responsible for their addition to growing glycoproteins have been localized. Such a translocation cannot occur without the aid of a lipid-soluble carrier due to the inability of the highly polar nucleotide sugars to diffuse through the non-polar lipid membranes. The findings of De Luca,

et al. (1973) indicate that vitamin A may be functioning in this capacity in the liver.

B. Plasma Glycoproteins

Most of the plasma proteins, with the exception of the immunoglobulins which are synthesized by the plasma cells (Winterburn and Phelps, 1972), are made by the liver. More than half of the liver's contribution to the plasma is comprised of the non-glycosylated proteins albumin, prealbumin and retinol-binding protein. The remainder contains sugar side-chains and represent the bulk (as a group) of the glycoproteins found in plasma.

The protein moiety of the glycoproteins is synthesized on the rough endoplasmic reticulum of the liver (Spiro, 1970). The sugar chains are built up in a step-wise fashion as the completed polypeptide moves from the rough endoplasmic reticulum, through the smooth endoplasmic reticulum, to the Golgi apparatus (Schacter, *et al.*, 1970). Some of the glycosyltransferases involved in this process are known to be associated with the particulate fraction of the endoplasmic reticulum (Spiro, 1970). The transferases responsible for the addition of the terminal sugars have been localized in the Golgi apparatus by Schacter, *et al.* (1970). It has been suggested that the terminating sugars, sialic acid or fucose, may be added immediately prior to secretion (Winterburn and Phelps, 1972).

The polysaccharide chains of plasma glycoproteins produced by the liver consist of a core of repeating mannose residues (the number varies from glycoprotein to glycoprotein) linked to N-acetylglucosamine, which is linked to the peptide chain by the formation of an N-glycosidic bond to asparagine (Spiro, 1970). To this core are added trisaccharide chains of

N-acetylglucosamine and galactose with fucose or sialic acid as the non-reducing, terminal sugar. The number of these branch chains, which are connected to a single core, varies depending on the particular glycoprotein, as does the density of polysaccharide side-chains with respect to the protein moiety.

The exact number of glycoproteins present in plasma is unknown, though there are believed to be more than a hundred (Buddecke, 1972). Freeman and Smith (1970) have characterized 61 distinct proteins in a fractionation of human serum on Sephadex G-150. When expressed as mg per 100 ml, thirteen glycoproteins plus the Immunoglobulins account for over 98% of the total concentration of glycoproteins in human serum (Shultze and Heremans, 1966).

The plasma glycoproteins have been grouped, based upon their electrophoretic mobility, into four classes, $alpha_1$, $alpha_2$, beta and gamma. The density of sugar on a glycoprotein seems to be the major determinating factor for the observed mobility. For example, the $alpha_1$ group, which exhibits the greatest migration at pH 8.6, also possesses the highest specific activity, when *in vivo* labelling with radioactive glucosamine is carried out in rats (Robinson, *et al.*, 1964). This direct correlation between electrophoretic mobility and apparent sugar density holds for the other three classes as well, with the gamma-globulins moving the slowest and having the lowest specific activity. Bekesi and Winzler (1967) found the same relationship when fucose was used as the labelled precursor in rats.

Changes in the blood levels of plasma glycoproteins occurs under a variety of circumstances. Several glycoproteins, known to respond to certain changes in the body, are called the "acute phase glycoproteins" (Winzler and Bocci, 1972). Jamieson, *et al.* (1972) have studied two such proteins,

alpha$_1$-acid glycoprotein and alpha$_2$-macroglobulin, in the rat following the induction of inflamation by turpentine injection. Another member of this group, haptoglobin, has been found to increase ten-fold under the same conditions (Sutton and Jamieson, 1972). This increase results from *de novo* synthesis by the liver, and not by a decrease in the rate of catabolism. Alpha$_2$-macroglobulin levels decrease in multiple myeloma and rheumatoid arthritis and increase in mongolism, telangiectasic ataxia and lipoidic nephrosis (Bourrillon and Razafimahaleo, 1972). The immunoglobulins exhibit rising levels following trauma, burns, infections and a variety of other conditions (Winzler and Bocci, 1972).

The function of the sugar side-chains found on glycoproteins has puzzled investigators for many years. Carbohydrate residues can be removed from plasma glycoproteins without affecting either transporting functions or tertiary structure (Winterburn and Phelps, 1972). Eylar (1964) proposed that the carbohydrate chains were a signal to the cell that the protein was destined for secretion. Winterburn and Phelps (1972) question this hypothesis based on various pieces of evidence. Primary among these is the recent report by Morrell, *et al.* (1971), which suggests that the sugar residues, minus sialic acid, represent a signal for the catalysis of the glycoprotein. The coded sequence and branching is presumably recognized by receptors in the liver plasma membranes, which bind the fated glycoprotein for transport to the lysosomes, where catabolism occurs. When desialylated glycoproteins are injected into healthly animals, they rapidly disappear from the plasma and are found to be trapped by the parenchymal cells of the liver. Drastically reduced half-lives are recorded for glycoproteins which have had their sialic residues removed. This hypothesis implicates neuraminidase activity in the plasma as the factor responsible for the rate of glycoprotein breakdown; neuraminidase

removes sialic acid from the side-chain terminals. Sialic acid is believed to serve as a block which prevents recognition and binding of the glycoprotein by the catabolizing tissue (Winterburn and Phelps, 1972).

C. Vitamin A and the Synthesis of Plasma Glycoproteins

As already noted, vitamin A has been found to affect the synthesis of glycoproteins in a variety of epithelial tissues (Levinson and Wolf, 1972; Prutkin, 1968; De Luca, *et al.*, 1971; De Luca and Wolf, 1972). Investigators were originally led to study glycoprotein production in these tissues by their observed degeneration in vitamin A-deficiency (Wolbach and Howe, 1925). In the liver, however, histological studies revealed little or no change in this organ in vitamin A-deficient animals, when compared to the livers of normal controls (Wolbach and Howe, 1925; Beaver, 1961). As a result, studies in the liver have been for the most part limited to the nature of the storage of vitamin A and its catabolism in this organ. It was not until Beaver (1961) observed tissues from vitamin A deficient rats raised in a germ-free environment that investigations were undertaken to link vitamin A to the normal functions performed by this organ.

Beaver's findings indicated that the liver of rats was indeed affected by vitamin A-deficiency, when these animals were isolated, and hence protected from infection by microorganisms. On the surface, he found that germ-free rats continued to grow beyond the time when normal vitamin A-deficient rats would begin to lose weight. Food intake was not decreased, when compared to normal controls, though a slower growth rate, ultimately levelling-off, indicated that the efficiency of absorption by the intestine was decreased in deficiency. (While these rats reached a weight plateau, weight loss did not occur as it does with normal vitamin A-deficient rats.) Germ-free

animals lived longer on the average than normal vitamin A-deficient rats, but this prolongation of life was not great. The surprising results of these studies were found when the livers of germ-free deficient animals were observed histologically after death. Beaver noted an extreme focal necrosis of the liver, "sufficient to cause death." While he declined speculation on the cause of this extensive tissue damage, it seems clear that vitamin A-deficiency has a profound effect on the liver, which can only be observed histologically after the time germ-infected rats would die.

Since Beaver's report appeared, several studies have been conducted on the production of plasma glycoproteins in vitamin A-deficient rats. Chagovets and Dusherko (1962) reported decreased incorporation from labelled methionine into the protein fractions of deficient-rat serum. Vakil, *et al.* (1964) and McLaren, *et al.* (1965) both found increased protein concentrations in plasma in vitamin A-deficiency, and specifically large increases in the gamma-globulin fraction. Plasma levels were determined by measuring protein-staining densities after paper electrophoresis and by assaying for total nitrogen in the work of Vakil, *et al.* (1964). Albumin levels were found to decrease in both studies. The findings of De Luca, *et al.* (1973) point to a possible mode of action for vitamin A in the liver, which could account for some of these observations.

INTRODUCTION

The changes in the levels of plasma glycoproteins reported by McLaren, *et al.* (1965) and Vakil, *et al.* (1964) in vitamin A-deficient rats are difficult to interpret in looking for a possible function for vitamin A with respect to these compounds. An increase in the plasma levels of a glycoprotein could either be due to an increase in its biosynthesis or a decrease in its rate of catabolism. At the same time, a decrease in the plasma volume with no change in either catalysis or synthesis would produce the same apparent increase in glycoprotein levels. As the hematocrit is increased in vitamin A-deficiency (McLaren, *et al.*, 1965), some rise in the concentration of glycoproteins is very likely associated with loss of fluid from the blood. Without firm data on changes in the synthesis and catalysis rates of plasma glycoproteins in vitamin A-deficiency, such evidence on protein levels in plasma lends itself only to idle speculation.

The formation of a retinylphosphate-mannose compound in the liver, studied by De Luca, *et al.* (1973), suggests that vitamin A might function in the transport of nucleotide sugars across the membranes of the Golgi apparatus or the endoplasmic reticulum. In this capacity, vitamin A would be carrying sugar residues from the cytoplasm to the site of their addition to the growing carbohydrate chains of glycoproteins. If vitamin A is involved in this process, glycoprotein synthesis would be expected to be depressed in the livers of vitamin A-deficient animals. As other polyprenols, specifically dolichol, found in the liver, form similar "sugar-lipid" compounds (Richards, *et al.*, 1971), it is probable that the glycosyltransferases responsible for the addition of sugar residues to synthesizing glycoproteins, exhibit specificity for both the various lipid carriers and the glycoproteins with which they react.

Various investigations, carried out in epithelial tissue from vitamin A-deficient rats (e.g. De Luca, *et al.*, 1970), have linked vitamin A to the synthesis of glycoproteins in these tissues. It seems reasonable to suspect that vitamin A might be involved in glycoprotein synthesis in the liver as well.

If vitamin A serves as a sugar carrier linked to the biosynthesis of glycoproteins by the liver, some effect should be registered on the rate of synthesis of at least some of these compounds in vitamin A-deficiency. Since the liver represents the major site for the synthesis of the plasma glycoproteins, the plasma presents itself as a convenient area in which to monitor glycoprotein production by the liver. In the studies being reported in this thesis, the rate of synthesis of the various fractions of the plasma glycoproteins was compared for normal and vitamin A-deficient rats to see if any differences in synthesis rate could be detected.

METHODS AND MATERIALS

A. Preparation of Animals

Weanling (21 days old) male albino rats of the Holtzman strain were obtained from Holtzman, Madison, Wis. Vitamin A-deficiency was brought about using the method of Wolf, *et al.* (1957); the vitamin A-free diet appears in Table I. Normal controls received the same diet plus 2,000 IU of vitamin A acetate per week. After four weeks, animals were paired (normal-deficient) according to weight and pair fed from then on. Pairs were chosen for use in studies when the deficient rats reached the weight-plateau stage. All experimental rats were switched to a vitamin A-free, 78% casein diet (Table II) two days before their use to ensure an adequate supply of amino acids for protein synthesis by the liver (Levi, *et al.*, 1968). Deficient animals showing symptoms of infection or diarrhetic animals were discarded as were their pairs. On the day of the experiment, rats had access to water only.

B. Obtaining Plasma for Pilot Studies

Rats were injected intraperitoneally with a known amount of a radioactively labelled sugar in 0.9% NaCl solution. Blood was taken from the tail vein at predetermined times (approximately 0.2 ml) and transferred to a 10 ml test tube containing 0.8 ml of a 0.9% NaCl solution with 0.01 M ethylenediaminetetraacetic acid (EDTA, to prevent clotting) at pH 7.0. Red cells were pelletted by centrifugation in a clinical centrifuge for 15 minutes. The supernatant (plasma) was removed with a tuberculin syringe to determine its volume and transferred to a 10 ml test tube at 4^{o} C.

TABLE I

Vitamin A-free Diet

Ingredients	*Amount ingredient added 1000 g total diet*
Heat-treated casein[a]	240 g
Sucrose	659 g
Salt mix 446[b]	50 g
Cotton seed oil	30 g
Choline dry mix[c]	10 g
Water-soluble vitamin mix[d]	10 g
Water-soluble vitamin D preparation[e]	1 ml
Vitamin E solution[f]	10 ml

[a]Heat-treated casein prepared by heating Labco vitamin-free casein in shallow pans at 105° C in a dry oven for 5 days

[b]From Spector (1948)

[c]20% choline premixed in cornstarch

[d]100 grams of water-soluble vitamin mix contained 250 mg of thiamine hydrochloride, 150 ml of riboflavin, 400 mg of calcium pantothenate, 1000 mg of nicotinic acid, 60 mg of pyridoxine hydrochloride, 60 mg of biotin, 40 mg of folic acid, 40 mg of 2-methyl-1,4-naphthoquinone, 0.5 mg of vitamin B_{12}, 2000 mg of inositol, 60 mg of p-aminobenzoic acid, and cellulose to make 100 g

[e]Water-soluble vitamin D preparation contained 2500 units per ml and was obtained from Endo Products, Inc., Richmond Hill, New York

[f]Vitamin E solution contained 60 mg of alpha-tocopherol per ml of petroleum ether

TABLE II

78% Casein Diet without Vitamin A

Ingredients	*Amount ingredient added 1000 g total diet*
Heat-treated casein	780 g
Roger's-Harper's Salt Mix	50 g
Vitamin mix 20[a]	20 g
Cotton seed oil	150 g
Choline[b]	15 ml
Vitamin B_{12}[c]	5 ml

[a]100 grams of vitamin mix 20 contained 50 mg of mendione, 20 mg of riboflavin, 40 mg of thiamine hydrochloride, 20 mg of pyridoxine hydrochloride, 100 mg of calcium pantothenate, 50 mg of folic acid, 250 mg of nicotinic acid, 1250 mg of inositol, 30 mg of vitamin D_2 (5000 IU/mg), 2250 mg of Roche vitamin E mix, and sucrose to make 100 grams.

[b]Choline solution was 20% (weight to volume) in water

[c]Vitamin B_{12} was 0.1% in water

C. Precipitation with 10% Trichloroacetic Acid

Plasma samples were made up to 10% trichloroacetic acid (TCA) by the addition of cold 50% TCA in water. Samples were allowed to precipitate overnight at 4° C. Precipitated protein was pelletted by spinning in a clinical centrifuge for 30 minutes. The supernatant was drawn off and saved to check the level of TCA-soluble radioactivity. 2.0 ml cold 10% TCA were added to each tube, and the pellet was resuspended. Samples were again allowed to stand overnight at 4° C, centrifuged and the supernatant removed. The pellet was washed twice with cold 10% TCA and stored at 4° C.

D. Protein and Radioactivity Determinations of Precipitates

Two different procedures were followed for determining the protein and radioactivity contents of the TCA precipitates:

1. Pellets were suspended in 1.0 ml of 0.05 M Tris-HCl buffer at pH 7.6. 0.1 ml of each suspension was added to 1.0 ml of NCS in a scintillation counting vial and digested by heating for two hours in a water bath at 50° C with occasional shaking. After digestion, 10.0 ml of toluene with .5% PPO was added to each vial. Vials were allowed to stand overnight in the dark before counting. Aliquots from the Tris suspensions were used for determination of protein concentration.

2. Pellets were suspended in 1.0 ml of 0.2 N NaOH and digested by placing the test tubes in a water bath at 80° C for one hour. 0.1 ml. aliquots were taken and counted for radioactivity. Aliquots from the same NaOH digestion were used for protein determination.

E. Obtaining Labelled Plasma for Fractionation

Rats received an intraperitoneal injection of radioactively labelled sugar

(either D-glucosamine or D-mannose) in 1.0 ml of a 0.9% NaCl solution. Deficient and normal rats received the same sugar, but labelled with different isotopes (^{3}H or ^{14}C). After three hours, rats were anesthesized with ether and blood was taken by cardiac puncture using the following procedure: rats were spread out on a ligation board; a 10 cc syringe with an 18 gauge needle was used; the needle was introduced between the sternum and the left rib cage and passed through the heart; slight suction was applied and the needle slowly withdrawn until blood flowed into the syringe. The blood obtained was quickly transferred to a large test tube containing 4.0 ml of 0.9% NaCl with 0.05 M EDTA pH 7.3 and thoroughly mixed. Blood was distributed equally between two graduated 15 ml conical centrifuge tubes and centrifuged for 30 minutes in a clinical centrifuge to pellet the red cells. Total volume, packed cell volume and plasma volume were determined and recorded for each animal, and the plasma transferred to a clean test tube at 4^{o} C.

F. Plasma Preparation for Fractionation on DEAE-Sephadex A-50

Plasma samples from deficient and normal rats were pooled separately and dialysed against two liters of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.001 M cold glucosamine. Dialysis was carried out for two days at 4^{o} C in glutathione-treated dialysis tubing with one change of buffer. (Glutathione treatment: dialysis tubing was allowed to soak for 3 hours in a solution of glutathione in water, then rinsed thoroughly with double-distilled water.) Following dialysis, aliquots were taken from each pool and radioactivity and protein concentration were determined. Volumes from each pool containing equal amounts of protein were mixed for fractionation and stored at 4^{o} C.

G. Fractionation on DEAE-Sephadex A-50

DEAE-Sephadex A-50 was swelled in excess 0.05 M Tris-HCl buffer, pH 8.0 (unless otherwise noted, "buffer" refers to this Tris buffer). Buffer was changed at least three times before use. A 2.5 x 60 cm column was carefully poured under gravity (bed volume approximately 300 ml). A 0.5 cm layer of Sephadex G-25 (coarse), swelled in the same buffer, was added to protect the bed surface. The column was equilibrated by running two bed volumes of buffer at a constant flow rate. Buffer was removed to the bed surface, and the mixed plasma sample carefully applied and run into the bed under gravity. Starting buffer was replaced, and a linear gradient was run from 0-0.6 M NaCl in buffer; identical reservoirs contained 800 ml each. A pump was used to maintain a constant flow rate, and fractions were collected every 10 minutes. Flow rate and other information accompanies the figures under *Results and Discussion.*

H. Preparation of samples for Gel Filtration

Samples for gel filtration were concentrated from approximately 10 ml to 1-2 ml by pressure dialysis (ultrafiltration) as described by Freeman (1970). Glutathione-treated dialysis tubing was used, and the surface area was kept at a minimum to limit loss of material. Following concentration, small (5 u.l.) aliquots were counted to determine the total DPM of each isotope contained in the sample.

I. Chromatography on Sephadex G-150 or Sepharose 6B

Sephadex G-150 (superfine) or Sepharose 6B were prepared by swelling in excess buffer with frequent changes over five days. Columns were carefully poured under gravity; individual column sizes used are reported in *Results.*

Void volume was determined by running a small amount of Blue Dextran 2000 with buffer wash maintained at a constant flow rate with a pump; collected fractions were measured for optical density (OD) at 254 nm, and the elution volume of the first peak was taken to be the void volume. These are reported with their respective columns under *Results*. Columns were further washed by running at least two bed volumes of buffer at constant flow. Sample application was achieved under gravity flow after removing excess buffer to the bed; buffer was replaced after sample application. Columns were connected to a buffer reservoir, and two bed volumes were run at the same flow rate as used for equilibration. Fractions were collected at timed intervals and stored at 4^{o} C until counted.

J. Preparation of Samples for Gel Electrophoresis

Samples were dialyzed against 1.0 liter of double-distilled water in glutathione-treated dialysis tubing to remove most of the salt. This was done to prevent denaturation during concentration. Dialyzed volumes were then concentrated in a rotary-flash evaporator (Buchler Instruments) until 0.25 ml of the sample contained at least 3,000 CPM of each isotope. An equal volume of 2.0 M sucrose in 0.025 M Tris-HCl buffer (pH 8.3) containing 0.17 M glycine was added to each sample to increase the density.

K. Disc Electrophoresis in 5% Polyacrylamide Gels

The method and materials used for polyacrylamide gel electrophoresis (P.A.G.E.) were based on Davis (1964) and Ornstein (1964). Stock solutions, buffers and the ratios in which they were combined to produce the 5% lower gel and the 2.5% stacking gel can be found in Table III. Gels were polymerized in 12 x .7 (i.d.) cm columns; 2.0 ml of the lower gel and 0.5

TABLE III

Preparation of 2.5% and 5% Polyacrylamide Gels

2.5% Upper Gel			*5.0% Lower Gel*		
Stock Solutions:		**Conc./100ml**	**Stock Solutions:**		**Conc./100ml**
	1 M H_3PO_4	16.0 ml		1 M HCl	30.0 ml
B:	Trisma Base	3.56 g	A:	Trisma Base	22.7 g
	TEMED[a]	0.2875 g		TEMED	0.2875 g
D:	Acrylamide	12.5 g	C:	Acrylamide	37.5 g
	"Bis"[b]	3.125 g		"Bis"	1.0 g
E:	Riboflavin		AP:	Ammonium persulfate	0.7 g
Mixing:	pH: 6.5-6.7		**Mixing:**	pH: 8.7-8.8	
B:	1.0 ml		A:	3.0 ml	
D:	1.0 ml		C:	2.0 ml	
E:	1.0 ml		AP:	1.5 ml	
Water:	2.0 ml		Water:	8.5 ml	
	5.0 ml			15.0 ml	
Upper Tray Buffer:[c]		pH 8.3	**Lower Tray Buffer:**[d]		pH 8.9
	Trisma Base	6.0 g		Trisma Base	908 g
	Glycine	28.8 g		HCl (conc.)	about 25 ml
	Water to	1.0 L		Water to	4 L

[a]N,N,N,N', Tetramethylethylenediamine

[b]N,N, Methylenebisacrylamide monomer

[c]Diluted 1:10 before use

[d]Diluted 1:5 before use

ml of the stacking gel mixture were used. Three gels were prepared and 0.5 ml of the sample-sucrose-buffer were added to each column along with a drop of dilute bromophenol blue solution (tracking dye). Apparatus was carefully set up using the upper and lower tray buffers indicated in Table III, and electrophoresis was carried out under a constant potential difference of 170 volts DC (current was approximately 2 milliamps per column; polarity top to bottom was - to +). Columns were stopped when the tracking dye advanced to within 1 cm of the bottom of the gel; running times were recorded for each gel.

L. Staining and Counting of Polyacrylamide Gels

Gels were either stained for protein or carbohydrate or counted for radioactivity as follows:

1. ***Protein staining***: gel was placed in a beaker containing a solution of amido black in 7.5% acetic acid for 30 minutes. Destaining was accomplished using an Anelco gel destainer against 7.5% acetic acid;

2. ***Carbohydrate staining***: periodic acid-Schiff's (PAS) staining technique was used as modified for polyacrylamide gels by Zacharias, *et al.* (1969). This procedure is outlined in Table IV.

3. ***Gel counting***: the slicing, digestion and counting of gel slices was similar to that suggested by Basch (1968) and Zaltin and Hariharasubramanian (1970). Gels were cut into uniform (approximately 2 mm) slices and placed in counting vials along with 0.5 ml NCS solubilizer. Digestion was effected by heating for two hours at 50^{o} C in a water bath, after which the vials were allowed to cool. 10 ml of toluene with .5% PPO was added to each vial, and the vials were left overnight in the dark at room temperature before counting.

TABLE IV

Procedure for Staining Polyacrylamide Gels for Carbohydrate Using the Periodic Acid-Schiff's Method

Procedure	*Time*
1. Immerse in 12.5% TCA (25-50 ml/gel)	30 minutes
2. Rinse lightly with double-distilled water	
3. Immerse in 1% periodic acid in 3% acetic acid	50 minutes
4. Wash in double-distilled water with changes, 200 ml/gel	overnight
5. Immerse in Schiff's reagent in the dark	50 minutes
6. Wash with freshly prepared 0.5% metabisulfite in 0.1 N HCl three times for 10 minutes each (25-50 ml/gel)	30 minutes
7. Wash in double-distilled water with stirring and frequent changes until excess stain is removed	overnight
8. Store in 7.5% acetic acid	

M. Analytical Techniques

1. ***Protein determination***: a modification of the Folin-Phenol method of Lowry, *et al.* (1951) was used. The standard curve was generated using bovine serum albumin. OD was read at 750 nm using a Zeiss spectrophotometer (used for all measurements of optical density reported in this thesis).

2. ***Determination of salt concentration***: resistivity was measured using a Serfass Conductance Bridge Model RC M15; conductivity was computed using the formula 1/resistivity = conductivity. Salt concentration was arrived at by the use of a standard curve (conductivity and salt concentration show a linear relationship).

3. ***Liquid scintillation counting***: unless otherwise stated, counting was carried out in 10 ml of Bruno's without POPOP: xylene, 500 ml; dioxane, 1500 ml; methylcellusolve (ethylene glycol monomethyl ether), 1500 ml; naphthalene, 280 g; PPO, 35 g. A Beckman Model LS-250 liquid scintillation counter was used with corrections for spillover and efficiency based on the external standard ratio. Calculations for the major fractionations were facilitated by the use of a PDP-8S computer (Digital Equipment Corp.).

N. Materials

D-glucosamine-1-^{14}C (53.1 mC/mM), D-glucosamine-6-^{3}H (3.6 C/mM), D-mannose-1-^{14}C (35.9 mC/mM) and PPO were obtained from New England Nuclear. D-mannose-2-^{3}H (1 C/mM) and NCS solubilizer were from Amersham/Searle. The vitamin A-free diet was produced by General Biochemicals. DEAE-Sephadex A-50, Sephadex G-25 and G-150, Sepharose 6B and Blue Dextran 2000 were all obtained from Pharmacia. Trizma Base (Tris) and bovine serum albumin were from Sigma.

RESULTS AND DISCUSSION

A. Pilot Studies: Normal Rat Time-Incorporation Study

Introduction: Before setting out to label and fractionate significant quantities of plasma, it was necessary to determine the time after injection of maximum specific activity in the plasma proteins of the rats being studied.

Various authors have reported on the incorporation of radioactivity from injected labelled D-glucosamine into the plasma (or serum) of rats (Shetlar, *et al.*, 1964; MacBeth, *et al.*, 1965; Robinson, *et al.*, 1964). These studies report peak specific activities in the blood at times ranging from three to five hours.

The variation encountered in the literature is most likely due to the fact that each group used rats of a different strain and size. One group (Shetlar, *et al.*, 1964) studied glucosamine incorporation in male albino rats of the Holtzman strain weighing 220-275 grams and found that maximum specific activity was reached three hours after intraperitoneal injection. The strain and weight range of these animals corresponded to that of the rats chosen for my own work. A pilot study was undertaken to determine whether or not my rats showed similar peak incorporation times as well.

The rat chosen for this initial study was not prepared as outlined under *Methods*. Instead, a 260 gram normal rat, fed standard laboratory rat chow *ad libitum* until the day of the experiment, was used to more closely model the rats used by Shetlar, *et al.* The rat was injected intraperitoneally at zero time with 16.25 uC of D-glucosamine-1-^{14}C in 0.15 ml of a saline solution. The experiment followed the procedure outlined under *Methods* with blood being sampled at the following times: 90, 120, 150, 180, 210 and

240 minutes after injection.

Results: Precipitation with 10% TCA and determination of protein and radioactivity (NCS method) were carried out as described in *Methods*. These results appear in Table V along with the calculated specific activities and the amounts of TCA-soluble radioactivity. The specific activities are plotted against time in Figure 1.

The graph shows two peaks, the first at 90 minutes and the second (maximum) at 180 minutes.

Using 3.5% of body weight as the volume of plasma with 7% of that being protein (Robinson and Winzler, 1964) the percentage of the original dose incorporated into plasma TCA-precipitable proteins at three hours was calculated. These assumptions, with the peak specific activity being 16.7 x 10^3 DPM/mg of protein, gave the following: plasma volume, 9.1 ml; plasma protein content, 637 mg; total incorporation into plasma protein, 10.6 x 10^6 DPM or 4.8 uC; percentage of original dose incorporated, 29.5%.

Table V shows that TCA-soluble radioactivity in the plasma was never more than 2% of the bound (precipitated) DPM. This is in agreement with the results found by other investigators (e.g. Shetlar, *et al.*, 1964).

A. Pilot Studies: Normal-Deficient Comparative Time Study

Introduction: It was important to know whether the pattern of incorporation or the peak time differed between vitamin A-deficient and normal pair-fed rats.

A normal-deficient pair of rats was prepared as outline in *Methods*. On the day of the experiment, the normal rat weighed 246 grams, and the deficient rat weighed 252 grams. Each rat received 62.5 uC D-glucosamine-6-^{3}H and 15.6 uC D-mannose-1-^{14}C in 0.25 ml of saline at zero

TABLE V

Plasma Protein, Bound and Free Radioactivity and Specific Activity for Normal Rat Time Study

	Precipitated with 10% Trichloroacetic Acid			*TCA[a] Soluble*	
Time	*Protein*	*DPM[b]*	*Specific Activity[c]*	*DPM*	*Ratio[d]*
90	4.05 mg	46,535	11,490	938.4	0.020
120	7.60 mg	79,438	10,452	703.8	0.009
150	7.75 mg	57,992	7,783	743.4	0.012
180	3.70 mg	61,974	16,750	613.3	0.010
210	4.65 mg	59,912	12,884	605.8	0.010
240	6.33 mg	74,952	11,840	840.4	0.011

[a]Trichloroacetic acid

[b]Disintegrations per minute

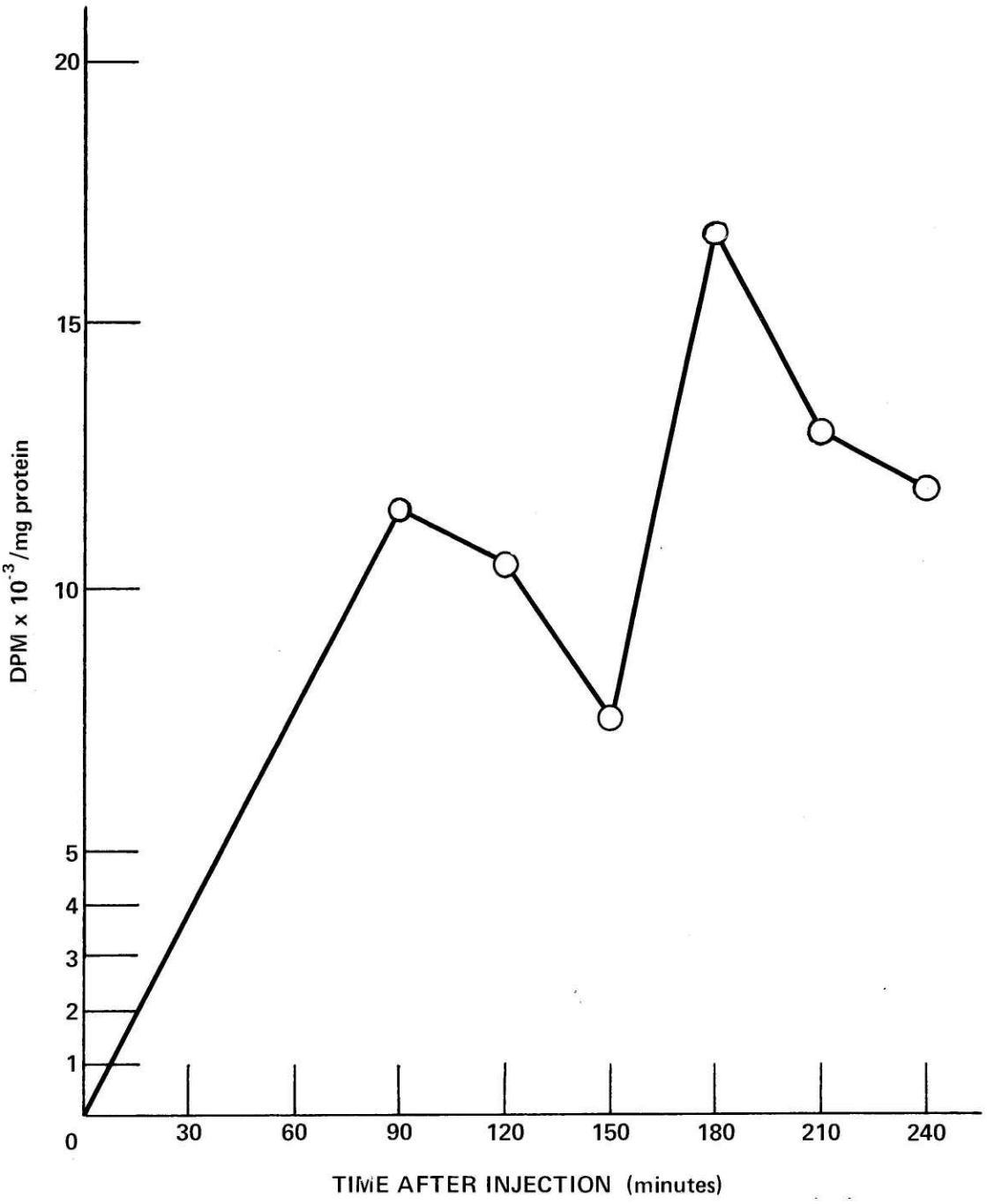
[c]Specific activity = DPM/mg protein

[d]Ratio of TCA soluble DPM to TCA precipitable DPM

FIGURE 1

Normal Rat Time Study:

Specific Activity vs. Time



time. The experiment was carried out as described in *Methods*, with blood being sampled at the following times: 60, 90, 120, 150, 180, 210 and 240 minutes after injection.

Results: TCA precipitation and digestion with 0.2 N NaOH were done as outlined in *Methods*. These results plus the calculated specific activities for each isotope can be found in Table VI. The specific activities were plotted as before (Figure 2).

From Figure 2, there appears to be no significant difference in the incorporation patterns for vitamin A-deficient and normal rats. The peak incorporation time is about three hours for both glucosamine and mannose.

Calculating the percentage of the original dose incorporated from the injected glucosamine at 150 minutes, using the same assumptions as stated previously, gives 12% for the normal and 12.6% for the vitamin A-deficient rat. Incorporation from mannose is extremely low for both deficient and normal animals, being less than 1% in both cases.

TCA-soluble radioactivity was found to be less than 2% of that recovered in the precipitated material at all times for both animals.

A. Pilot Studies: Comparison of Blood Protein Concentration

Introduction: McLaren, *et al.* (1965) report increased hematocrits for the blood of vitamin A-deficient rats and an increase in the concentration of total protein. Vakil, *et al.* (1964) did not find a significant increase in the blood protein concentration in experiments with pair-fed rats which had lost weight for two consecutive days. It was decided to check for such changes in protein concentration with the Holtzman rats being used for these studies.

A normal-deficient pair was used for this pilot study. On the day of the experiment, the normal rat weighed 251 grams and, the deficient rat

TABLE VI

Plasma Protein, Bound Radioactivity and Specific Activity Data for Normal-Deficient Comparative Time Study

A. Normal Rat

Time	*Protein*	*DPM-^{3}H*[a]	*S.A.*[b]*-^{3}H*	*DPM-^{14}C*	*S.A.-^{14}C*
60	5.44 mg	64,736	11,900	1137.0	209.0
90	8.88 mg	198,024	22,300	3063.6	345.0
120	8.84 mg	170,612	19,300	2351.4	266.0
150	12.40 mg	338,520	27,300	5084.0	410.0
180	8.56 mg	212,288	24,800	3398.3	397.0
210	16.60 mg	478,080	28,800	7121.4	429.0
240	7.37 mg	213,440	29,000	3312.0	450.0

B. Deficient Rat

Time	*Protein*	*DPM-^{3}H*	*S.A.-^{3}H*	*DPM-^{14}C*	*S.A.-^{14}C*
60	3.84 mg	29,600	7,710	430.1	112.0
90	5.68 mg	92,016	16,200	1363.2	240.0
120	3.96 mg	84,744	21,400	1366.2	345.0
150	7.20 mg	201,600	28,000	3276.0	455.0
180	6.96 mg	132,936	19,100	3354.7	482.0
210	7.36 mg	189,152	25,700	3547.5	482.0
240	7.44 mg	195,672	26,300	3355.4	451.0

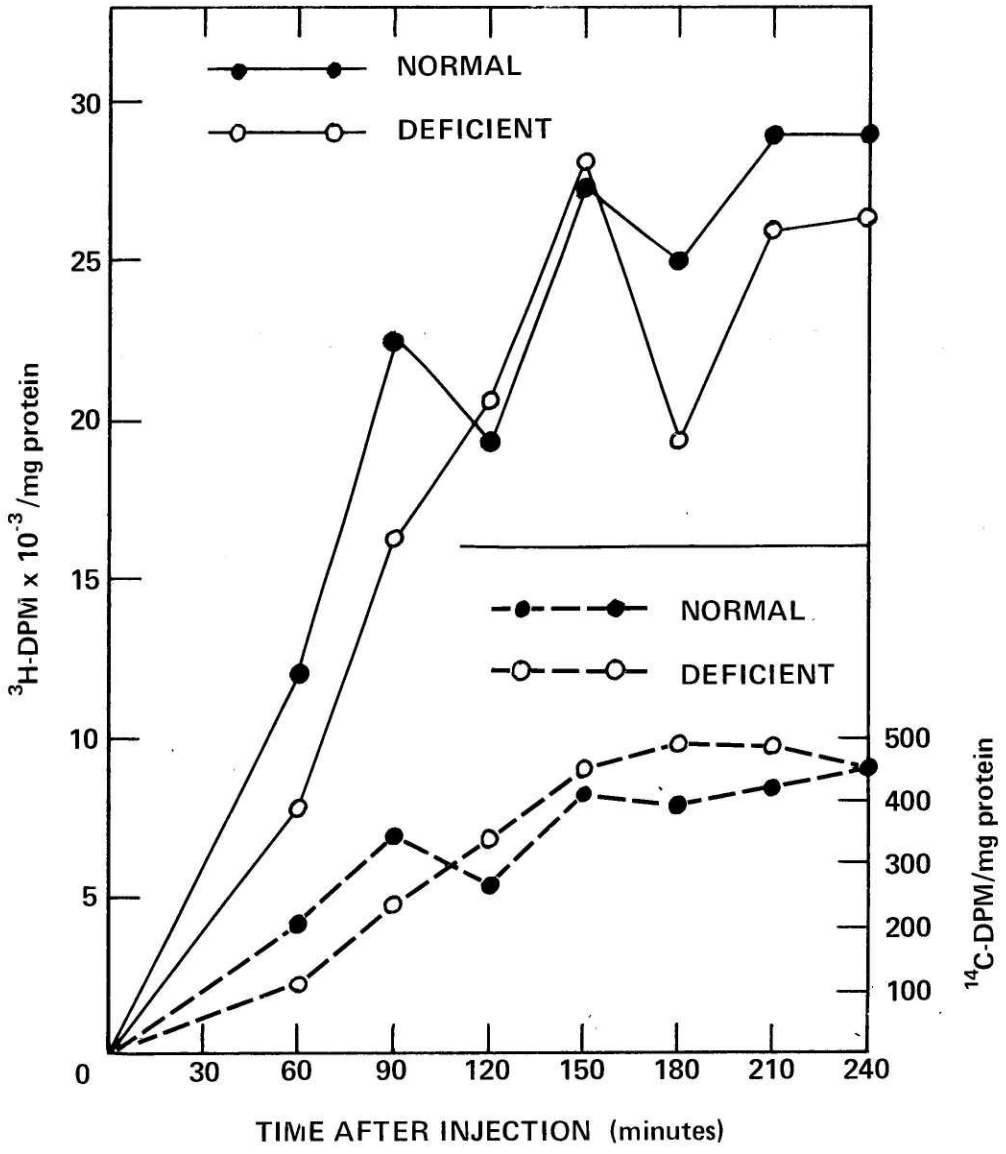
[a]Label incorporation into plasma glycoproteins is from D-glucosamine-6-^{3}H and D-mannose-1-^{14}C

[b]Specific Activity which is expressed as DPM/mg protein

FIGURE 2

Comparitive Time Study:

Specific Activity vs. Time



weighed 245 grams. Blood was taken by cardiac puncture, and the red cells pelletted as described under *Methods*.

Results: The hematocrit was found to be raised in vitamin A-deficiency; hematocrit results: normal, 45.1; deficient, 50.8.

Protein determination was carried out on the plasma samples; calculations were done to give the protein concentration in whole blood. The concentration of protein in the blood of the vitamin A-deficient rat was found to be higher: normal, 34.6 mg protein/ml whole blood; deficient, 37.3 mg protein/ml whole blood. For plasma, these values were even further separated: normal, 64.0 mg protein/ml plasma; deficient, 75.8 mg protein/ml plasma.

A. Pilot Studies: Discussion

Time-incorporation studies: All of the incorporation studies encountered in the literature used a large number of rats. Rats were sacrificed to obtain blood in quantity, and the data from at least three animals were averaged for each time point. The results from these studies produce smooth incorporation curves, rising rapidly to their peak and then dropping gradually as the labelled glycoproteins are removed from circulation and degraded.

The extremely small scale of the two studies reported is most likely responsible for the lack of smoothness in the final graphs (Figures 1 and 2). However, the purpose was not to generate immaculate curves and unimpeachable peak times but 1) to establish the approximate time of maximum incorporation in the plasma of the experimental rats being used and 2) to show that there is no significant difference between either the time of peak incorporation or the general pattern of incorporation in vitamin A-deficient and normal pair-fed rats. With this in mind, Figures 1 and 2

represent valid templates from which the required information can be extrapolated. Visualizing smoother curves, the following conclusions can be drawn:

1. The time of maximum incorporation for rats of the size and strain being used in these studies is approximately three hours;

2. The peak time and incorporation pattern does not vary between chow-fed normal rats and those prepared as described under *Methods*;

3. There is no significant difference between normal and vitamin A-deficient pair-fed rats in peak time, pattern or amount of incorporation.

The results obtained for the chow-fed rat agree well with those reported by Shetlar, *et al.* (1964) for similar rats. They found a peak at three hours and a maximum incorporation of 24% of the administered dose. In addition, the low figures for TCA-soluble radioactivity (i.e. free sugars) correlate well with those found by other authors.

While the peak specific activity increased with the increased dose (in uC) given in the second study, the percentage of the injected radioactivity incorporated into the total protein in plasma as calculated was less. The reason for this decreased percent of incorporation is not clear. The literature shows wide variation on this point with as much as 37% incorporation into plasma glycoproteins (Robinson, *et al.*, 1964) reported in some studies. Comparing specific injections used in these various studies does not clarify the situation: 15.5 uC in 1.3 mg, 24% (MacBeth, *et al.*, 1965); 20.0 uC in .5 mg, 24% (Shetlar, *et al.*, 1964); 4.0 uC in 1.2 mg, 37.5% (Robinson, *et al.*, 1964); 16.25 uC in 0.046 mg, 29.5% (normal rat time study); 62.5 uC in 0.0025 mg, 12.0% (pair-fed normal rat) (all of these studies used glucosamine-1-^{14}C except the normal pair-fed rat experiment, which used glucosamine-6-^{3}H). It would seem from these results that most sugar-least

radioactivity results in the highest percentage of radioactivity incorporation; the exact opposite results in the lowest percentage. However, the data is far from conclusive between these two extremes. Most likely, amount of uptake into the plasma glycoproteins is mediated by a host of factors including strain, weight, metabolic state and type of injection given. The observed variation is clearly *not* due to the liver pool size!

Blood protein study: Protein concentration in whole blood shows an 8% increase in vitamin A-deficiency; an 18% increase can be calculated for the protein concentration in plasma. These figures are lower than those found by McLaren, *et al.* (1965), but in disagreement as well with Vakil, *et al.* (1964), who saw no change at all in vitamin A-deficiency. Of course, these results found here are far from significant, being based on one normal and one vitamin A-deficient rat. The change in the hematocrit is well established, and appears in the majority of the blood sampling data reported with the next series of experiments.

It is interesting to note that if one corrects for the plasma volume based on the change in the hematocrit for the deficient rat, the protein concentration remains increased in vitamin A-deficiency, though only by 6% in this experiment. One would like to be able to say that this is due to an increase in synthesis or a decrease in catalysis, however there is no firm evidence which would support either one of these.

B. Fractionation on DEAE-Sephadex A-50: Experiments I-IV

Introduction: Most previous studies, which compared plasma (or serum) protein levels by fractionation, have used unlabelled material with protein levels being monitored by measuring absorbance at 280 nm (e.g. Jamieson, *et al.*, 1972). Such comparisons require that two parallel fractionations be run, one for experimental plasma and the other for that from normal controls. These studies are, therefore, complicated by the fact that it is almost humanly impossible to set up two (or more) identical columns with identical flow rates, etc. As a result, the data obtained is more difficult to interpret accurately. For these reasons, it was decided to run normal and deficient plasma on the same DEAE-Sephadex column at the same time. By labelling with different isotopes of the same sugar, experimental and control levels could be observed separately while running under identical conditions. By reversing the isotopes and repeating the experiment, errors introduced in scintillation counting could be checked.

Several authors (e.g. Shetlar, *et al.*, 1964) have established that radioative glucosamine, when injected into a rat, labels plasma glycoproteins mainly as hexosamine, and as sialic acid exclusively. It is not rapidly metabolized to CO_2 (approximately 1% per hour; Shetlar, *et al.*, 1964). No labelling of the amino acid found in plasma proteins occurs in the first three hours after injection (Robinson, *et al.*, 1964). In addition, the Immunoglobulins, which are glycoproteins made by the plasma cells and not by the liver, do not show significant uptake of label from injected sugars. Glucosamine, then, allows one to observe only the glycoproteins synthesized by the liver, as the non-glycosylated plasma proteins and the Immunoglobulins do not incorporate label from injected radioactive glucosamine. For these reasons, labelled glucosamine was used in three of the four plasma

fractionations performed.

One experiment was carried out using mannose as the labelled precursor. Mitranic and Moscarello (1972) have reported that injected mannose is rapidly converted to all other other sugars found in plasma glycoproteins, chiefly sialic acid and fucose. Peak incorporation as carbohydrate in plasma glycoproteins was found to be *5 minutes* after intravenous injection via the jugular vein, and this decreased rapidly to 120 minutes (longest time studied). My own work (Figure 2), which follows the time incorporation of ^{14}C from mannose-1-^{14}C into total plasma protein, indicates a peak incorporation time of three hours with a very low (less than 1%) percentage of original dose being incorporated. Whether or not this observed incorporation was as labelled mannose is not known. However, because of the high mannose content of plasma glycoproteins (Spiro, 1970) and the suggestion by De Luca, *et al.* (1973) that a metabolite of retinol might serve as a carrier of mannose in the synthesis of these glycoproteins by the liver, it was decided to use this sugar as an alternative marker.

For these experiments, normal-deficient pairs were prepared as outlined under *Methods*. Two pairs were used for each experiment. Rats were injected intraperitoneally at zero time with labelled sugar in 1.0 ml saline. Weight data, injected isotopes and amounts are given for each experiment in Table VII. Fractionation on DEAE-Sephadex A-50 was carried out as described under *Methods*.

Results: Blood sampling data for all four experiments is given in Table VIII; total blood volume obtained, and packed cell volume (PCV) and plasma volume after centrifugation, are recorded for each rat used.

After dialysis, but before mixing and application to the column, aliquots of pooled normal and pooled deficient plasma were taken to

TABLE VII

Animal Weights on Day of Experiment, Isotope Injected and the Amount Administered, Experiments I-IV

Experiment	*Rat*	*Weight*	*Isotope*	*Amount Injected*
I	Normal A	276 grams	glucosamine-6-^{3}H	75 u.C.
	Deficient A	250 grams	glucosamine-1-^{14}C	75 u.C.
	Normal B	253 grams	glucosamine-6-^{3}H	75 u.C.
	Deficient B	242 grams	glucosamine-1-^{14}C	75 u.C.
II	Normal A	262 grams	glucosamine-1-^{14}C	75 u.C.
	Deficient A	237 grams	glucosamine-6-^{3}H	75 u.C.
	Normal B	266 grams	glucosamine-1-^{14}C	75 u.C.
	Deficient B	223 grams	glucosamine-6-^{3}H	75 u.C.
III	Normal A	295 grams	mannose-2-^{3}H	25 u.C.
	Deficient A	265 grams	mannose-1-^{14}C	25 u.C.
	Normal B	312 grams	mannose-2-^{3}H	25 u.C.
	Deficient B	278 grams	mannose-1-^{14}C	25 u.C.
IV	Normal A	248 grams	glucosamine-1-^{14}C	75 u.C.
	Deficient A	227 grams	glucosamine-6-^{3}H	75 u.C.
	Normal B	243 grams	glucosamine-1-^{14}C	75 u.C.
	Deficient B	220 grams	glucosamine-6-^{3}H	75 u.C.

TABLE VIII

Blood Sampling Data, Experiments I-IV

Experiment	*Rat*	*Total Blood*[a]	*P.C.V.*[b]	*Plasma*
I	Normal A	7.3 ml.	3.5 ml.	3.8 ml.
	Deficient A	7.8 ml.	3.8 ml.	4.0 ml.
	Normal B	7.5 ml.	3.7 ml.	3.8 ml.
	Deficient B	7.1 ml.	3.5 ml.	3.6 ml.
II	Normal A	5.9 ml.	3.0 ml.	2.9 ml.
	Deficient A	7.5 ml.	4.0 ml.	3.5 ml.
	Normal B	7.2 ml.	3.6 ml.	3.6 ml.
	Deficient B	1.9 ml.	0.8 ml.	1.1 ml.
III	Normal A	4.6 ml.	2.1 ml.	2.5 ml.
	Deficient A	2.3 ml.	1.2 ml.	1.1 ml.
	Normal B	5.4 ml.	3.6 ml.	2.8 ml.
	Deficient B	5.0 ml.	3.3 ml.	2.7 ml.
IV	Normal A	7.3 ml.	3.6 ml.	3.7 ml.
	Deficient A	62 ml.	3.0 ml.	3.2 ml.
	Normal B	8.4 ml.	3.6 ml.	4.8 ml.
	Deficient B	8.4 ml.	4.0 ml.	4.4 ml.

[a]Volume does not include 4.0 ml. EDTA solution (see *Methods*)
[b]Packed Cell Volume

determine their specific activities. These appear in Table IX, along with the amount of protein and the total radioactivity from each pool that was applied to the column.

Aliquots from each fraction off DEAE-Sephadex were taken and counted for radioactivity. After correcting for spillover and efficiency, these results were plotted for each experiment (Figures 3-6). Selected fractions were used to check the linearity of the NaCl gradient and to establish the salt concentration at which the various peaks were eluted; these appear in the same figures as the radioactivity profiles (not done for Experiment III).

Comparing these radioactivity profiles (Figures 3-6) with electrophoretic results obtained with human serum on cleared cellulose acetate strips (Klainer, *et al.*, 1968) reveals that the patterns are essentially the same. (Klainer, *et al.* determined glycoprotein by staining for carbohydrate using the Periodic Acid-Schiff's method and measuring stain density with a recording densitometer.) Since the behavior of molecules on cellulose acetate and DEAE-Sephadex is predominately a function of charge, a comparison between these two separation systems is valid. By so doing, it is possible to identify the four peaks eluted from DEAE-Sephadex with the four plasma glycoprotein groups; from left to right (Figures 3-6), they are: gamma, beta, $alpha_2$ and $alpha_1$.

When compared among themselves, the elution patterns from the four experiments all have the same general appearance. All four indicate a depression in the peak designated as being the $alpha_1$ globulins. This peak is eluted at a NaCl concentration in 0.05 M Tris buffer of approximately 0.23 M NaCl. Summation of the deficient and of the normal radioactivity in this peak shows this depression to be about 30% in all four experiments; it is very likely that this depression is in actuality greater than 30%, but the

TABLE IX

Plasma Data Prior to Application

to DEAE-Sephadex A-50, Experiments I-IV

Experiment	*Plasma*[a]	*Specific Activity*[b]	*Mixed*[c]	*DPM*[d]
I	Normal-^{3}H	36,700	400 mg	1.47×10^{7}
	Deficient-^{14}C	33,800	400 mg	1.35×10^{7}
II	Normal-^{14}C		335 mg	
	Deficient-^{3}H		335 mg	
III	Normal-^{3}H	528.6	340 mg	1.80×10^{5}
	Deficient-^{14}C	435.0	340 mg	1.48×10^{5}
IV	Normal-^{14}C	28,090	600 mg	1.69×10^{7}
	Deficient-^{3}H	26,588	600 mg	1.59×10^{7}

[a]Source animal and the labelling isotope

[b]DPM/mg protein

[c]Volumes of plasma mixed for application to the column contained this amount of protein

[d]Amount of radioactivity applied to the column

FIGURE 3

Plasma Fractionation on DEAE-Sephadex A-50:

Experiment I (Glucosamine)-Normal, ^{3}H; Deficient, ^{14}C

Flow Rate: 24 ml/hour

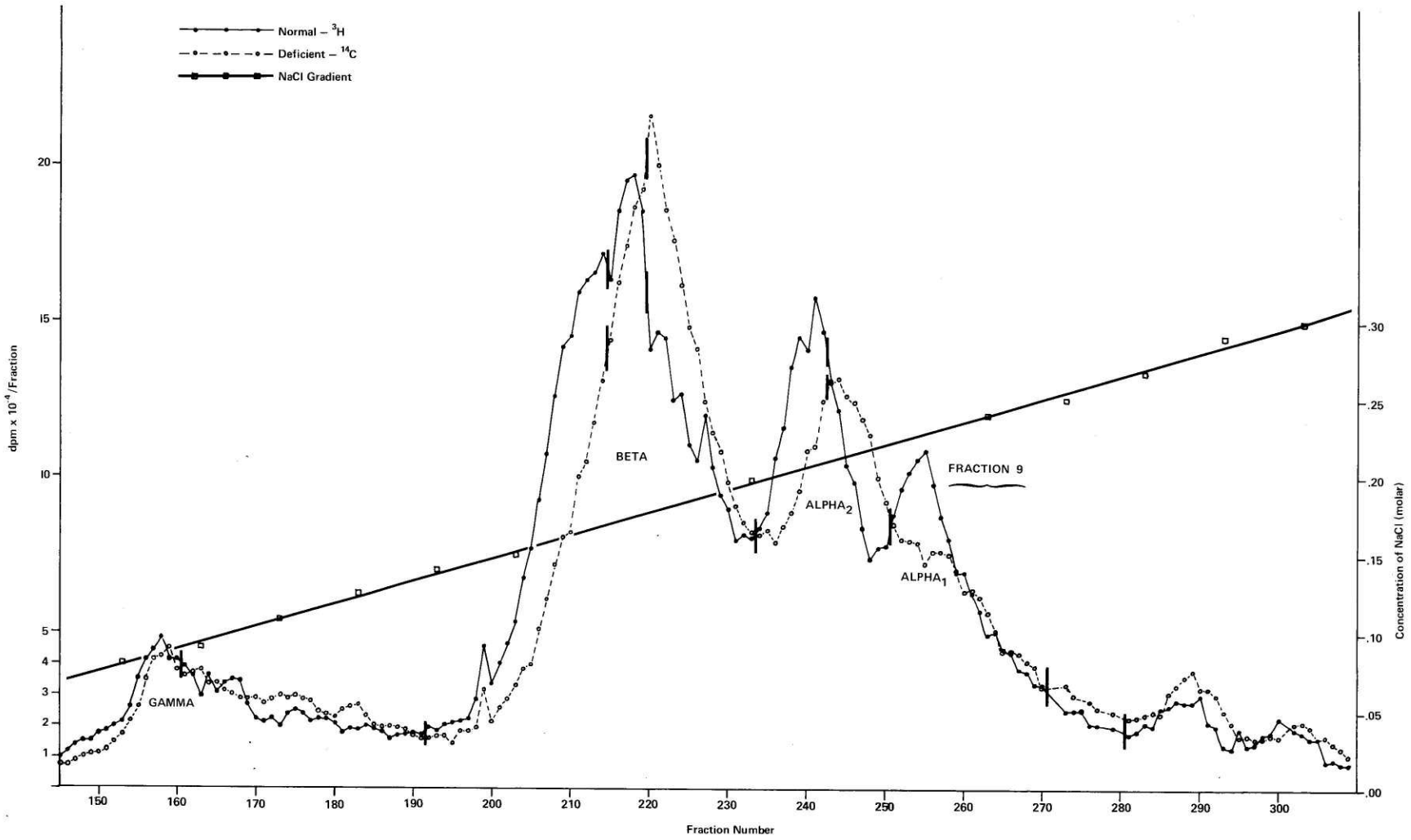

Normal – ^{3}H
Deficient – ^{14}C
NaCl Gradient
dpm x 10^{-4}/Fraction
Concentration of NaCl (molar)
Fraction Number
GAMMA
BETA
ALPHA$_2$
ALPHA$_1$
FRACTION 9
1
2
3
4
5
10
15
20
.00
.05
.10
.15
.20
.25
.30
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290
300

FIGURE 4

Plasma Fractionation on DEAE-Sephadex A-50:

Experiment II (Glucosamine)-Normal, ^{14}C; Deficient, ^{3}H

Flow Rate: 24 ml/ hour

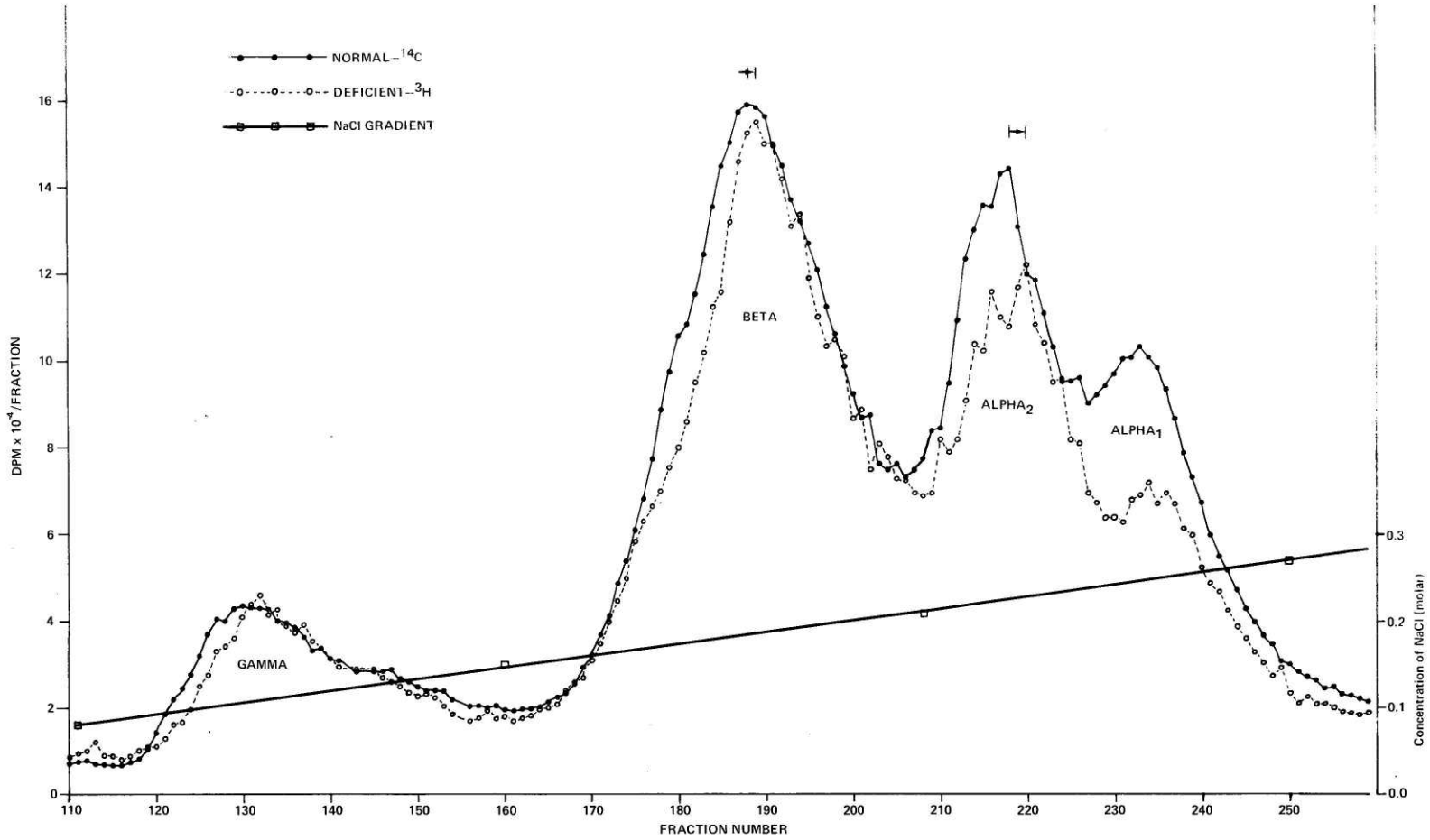

NORMAL--^{14}C
DEFICIENT--^{3}H
NaCl GRADIENT
DPM x 10^{-4}/FRACTION
Concentration of NaCl (molar)
FRACTION NUMBER
GAMMA
BETA
ALPHA$_2$
ALPHA$_1$
0
2
4
6
8
10
12
14
16
0.0
0.1
0.2
0.3
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250

FIGURE 5

Plasma Fractionation on DEAE-Sephadex A-50:

Experiment III (Mannose)-Normal, ^{3}H; Deficient, ^{14}C

Flow Rate: 24 ml/hour

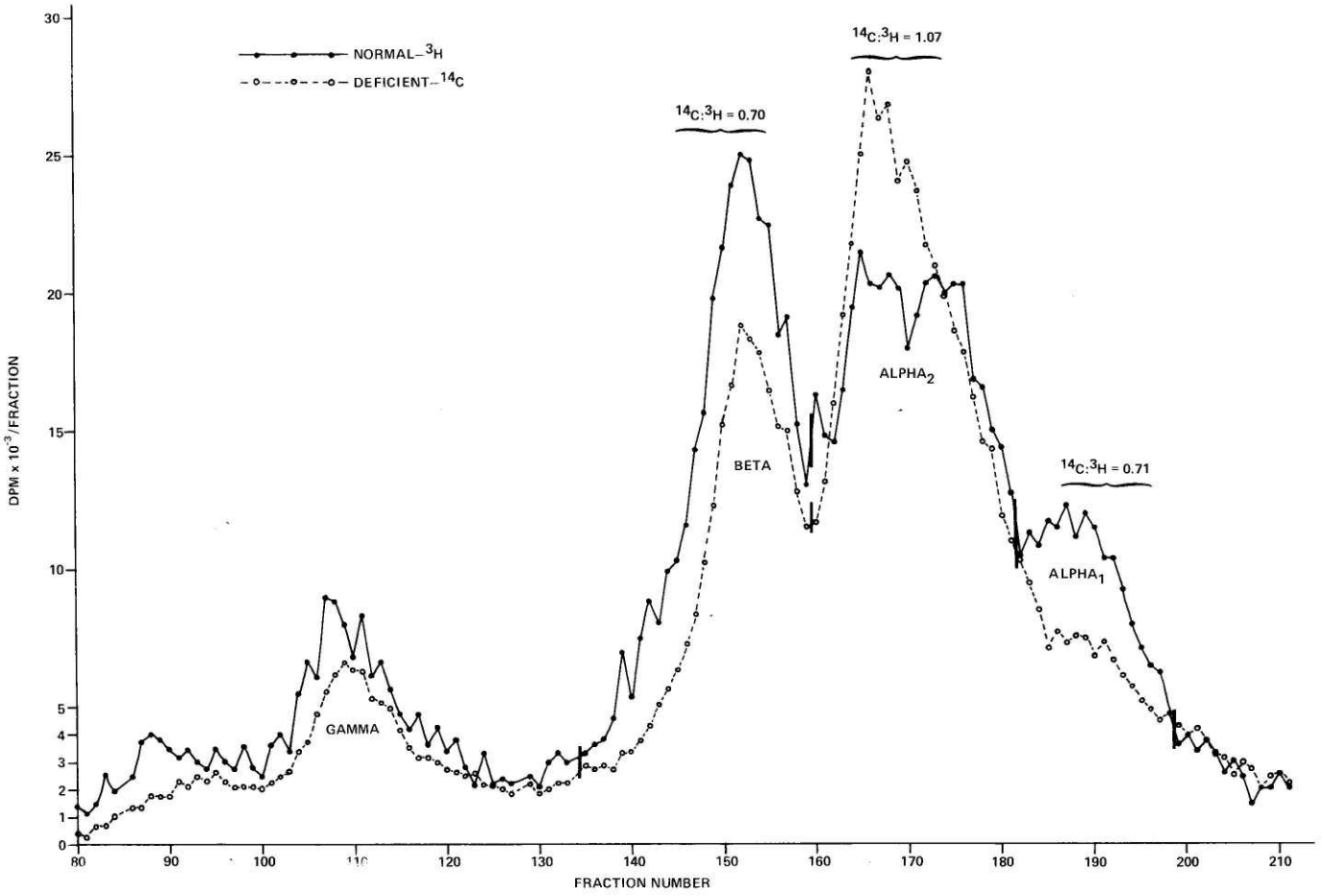
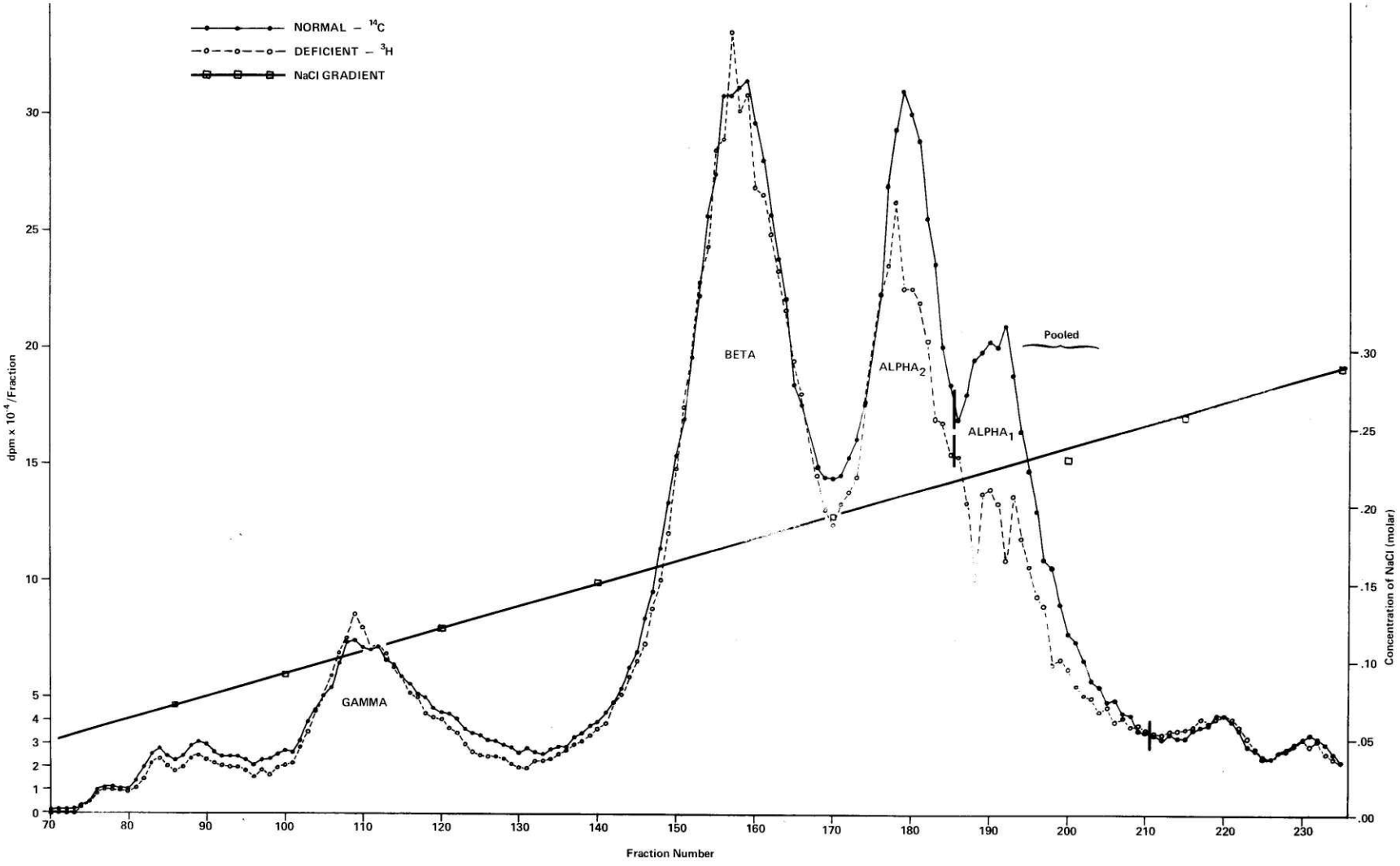

NORMAL–^{3}H
DEFICIENT–^{14}C
^{14}C:^{3}H = 0.70
^{14}C:^{3}H = 1.07
^{14}C:^{3}H = 0.71
BETA
ALPHA$_2$
ALPHA$_1$
GAMMA
DPM x 10^{-3}/FRACTION
FRACTION NUMBER
0
1
2
3
4
5
10
15
20
25
30
80
90
100
110
120
130
140
150
160
170
180
190
200
210

FIGURE 6

Plasma Fractionation on DEAE-Sephadex A-50:

Experiment IV (Glucosamine)-Normal, ^{14}C; Deficient, ^{3}H

Flow Rate: 30 ml/hour


NORMAL – ^{14}C
DEFICIENT – ^{3}H
NaCl GRADIENT
dpm x 10^{-4}/Fraction
Concentration of NaCl (molar)
Fraction Number
GAMMA
BETA
ALPHA$_2$
ALPHA$_1$
Pooled
0
1
2
3
4
5
10
15
20
25
30
.00
.05
.10
.15
.20
.25
.30
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230

trailing edge of the $alpha_2$ peak interferes in such a way as to make it appear to be less. The beta and gamma peaks show no effect in Figures 3, 4 and 6 (glucosamine label), however, when mannose is the labelled precursor, decreases are evident for the deficient profile in Figure 5. (Some of the singular aspects of the mannose experiment are discussed separately, below.) The $alpha_2$ peak from the three glucosamine experiments shows some depression in vitamin A-deficiency, though to a lesser degree than the $alpha_1$ material; experiment III with mannose would seem to show a stimulation of incorporation into the $alpha_2$ peak, however the deficient to normal peak ratio is actually only 1.07 for this peak (Figure 5).

Following the running of the gradient in experiment IV, the DEAE-Sephadex column was washed, first with 1.0 M NaCl in buffer and then with 2.0 M NaCl. Both of these step elutions gave peaks, however no difference in incorporation was found either initially or when the peaks were concentrated and re-run on smaller columns of DEAE-Sephadex using linear gradients (0.6-1.0M and 1.0-2.0 M NaCl).

The different specific activities of the two glucosamine isotopes used does not seem to affect the level of incorporation. This would indicate that the endogenous liver pools of this carbohydrate are large compared to the amount of sugar injected.

The deficient rat plasma consistently carried a lower specific activity (DPM/mg protein) than the normal plasma. It could be said that this is due to unchanged rates of synthesis for the beta-globulins, while the synthesis of the alpha-globulins has decreased. However, nothing is known about the levels of the non-labelled proteins in these experiments, and changes in these would definitely result in changes in the apparent specific activities, as the non-labelled plasma constituents account for over half of the total

concentration of protein in normal plasma.

Figure 3 from experiment I shows an obvious shift in the elution patterns for deficient and normal plasma. Since these are running simultaneously on the same column, there must be some difference which is causing the glycoproteins from vitamin A-deficient rats to be eluted later than the normal glycoproteins. This could either be due to a greater negitivity of the deficient glycoproteins or to some difference in molecular weight, since some filtration effects occur on DEAE-Sephadex. While it is less evident in experiment II, the shift seen in Figure 4 is in the same direction as that seen in experiment I. This would indicate that the observed effect is not an isotope effect, as the isotopes are reversed in these two experiments. No shift presents itself in the last two fractionations (Figures 5 and 6). For experiment IV (Figure 6), this disappearance of the shift could be explained by the higher flow rate used. The lack of an effect in experiment III (Figure 5) may be the result of the different labelling pattern of this sugar precursor as well as the much decreased incorporation; the deviation seen in the mannose elution pattern from that observed when glucosamine is injected suggests that comparisons between these two may not, in fact, be valid at all.

All columns shrank roughly 50% during the running of the gradient. While this greatly increased the mixing volume above the bed, the gradient remained linear throughout the region of fractionation as can be seen in Figures 3, 4 and 6.

The bed surface was disturbed after the sample was applied in experiment II. The surface was not horizontal while the fractionation was being run. As a result, some peak broadening is evident in Figure 4, though the elution pattern is essentially unchanged with respect to the other two

glucosamine experiments.

The profile from experiment III, which used mannose as the labelling sugar (Figure 5), differs from the others in that the $alpha_2$ peak shows the highest degree of label incorporation. Since mannose can be metabolized to other sugars in the liver, particularly glucose, some of the label that appears in the plasma could be associated with most of the other sugars normally found in glycoproteins, and in the amino acids as well. This would be expected to result in some difference, when compared to incorporation from glucosamine. The variation in the number of mannose residues contained in the core of the carbohydrate side-chains (Spiro, 1970) results in different mannose to N-acetylglucosamine ratios in different glycoproteins. This is probably the major cause of the observed differences between the mannose- and glucosamine-labelled fractionation profiles.

After analysis of the fractions from experiment I, peaks were pooled as indicated in Figure 3. The pooled number 9 Fraction was chosen for further fractionation. These pooled fractions were stored at 4^{o} C. Following experiment IV, the peak corresponding to Fraction 9 was also pooled and frozen as indicated in Figure 6; this material was not used in the experiments that follow.

B. Fractionation on DEAE-Sephadex A-50: Discussion

The results of these experiments indicate that differences exist between vitamin A-deficient and normal rats in the rate of incorporation of labelled sugar precursors into the plasma glycoproteins. Upon fractionation, the $alpha_1$ peak shows a consistent depression in vitamin A-deficiency of at least 30%; the $alpha_2$ peak is depressed to a lesser degree than the $alpha_1$, when glucosamine labelling is done, but shows an apparent stimulation when

mannose is the labelled precursor.

The cause of the shift in experiments I and II (Figures 3 and 4) was not investigated further, and thus remains a mystery. Since it is presumably not due to an isotope effect, some basic difference could be responsible for the observed difference. If the effect is caused by a difference in molecular weight, a higher degree of uncompleted sugar side-chains (known as microheterogeneity; Spiro, 1970) could be characteristic of the deficient plasma glycoproteins. If this shift is due to charge differences, a larger amount of sialic acid in the side-chains of glycoproteins from vitamin A-deficient-rat plasma would be the most likely explanation. This is because sialic acid contributes the most to the acidity of glycoproteins; the other sugar residues are uncharged at pH 8.0. Why the deficient glycoproteins would contain more sialic acid is puzzling, though it could be caused by a decrease in the neuraminidase activity in the plasma of vitamin A-deficient rats. A final possible explanation for the shift would be a change in the peptide chains themselves.

For these experiments, equal amounts of plasma protein from normal and vitamin A-deficient rats were compared. Protein was chosen as the baseline rather than equal plasma volumes or equal amounts of radioactivity for the following reasons:

1. Changes in the concentration of total protein in the plasma are not predictable. My own work indicates an increase in the concentration of protein in whole blood in vitamin A-deficient rats when compared to pair-fed controls, while Vakil, *et al.* (1964) report no change. McLaren, *et al.* (1965) finds a 22% increase in concentration in deficient rats, compared to the 8% increase that I found. Because these changes are probably caused by concentration due to the loss of fluid from the blood (decrease in total

blood volume; hematocrit increases in vitamin A-deficiency) as opposed to changes in the active synthesis and breakdown of glycoproteins, comparisons made on the basis of equal plasma volumes would be very difficult to interpret. If it were possible, comparison based on total plasma volume would be the best choice.

2. The use of radioactivity as the baseline for comparison was considered, however, it was decided that general increases or decreases in synthesis or effects due to differences in the free sugar pools of the liver would be harder to spot.

The use of protein as the basis for comparison is not free from complications. Large changes in the levels of the non-glycosylated plasma proteins, cheifly albumin, and the Immunoglobulin (which are not significantly labelled when radioactive labelling is done with glucosamine; MacBeth, *et al.*, 1965) could cause changes in the percentage of total labelled glycoprotein with respect to total plasma protein. Such changes would be expected to result in an equal shifting, up or down, of the entire pattern. Vakil, *et al.* (1964) found a decrease in albumin and an increase in the gamma-globulins (expressed as percent of total protein), when comparing pair-fed normal and vitamin A-deficient rats. (The gamma-fraction is dominated by the Immunoglobulins; Winzler and Bocci, 1972.) The decrease of albumin levels and the increase in the gamma-fraction effectively cancel each other out according to the findings of Vakil, *et al.*, leaving the alpha- and beta-globulins at approximately normal levels. Since the alpha- and beta-globulins are the main contribution of the liver, and are therefore the glycoproteins of most interest in my studies, comparison based on equal amounts of plasma protein appears to be a valid one, though not unquestionably so.

C. Fractionation of Pooled Fraction 9: Gel Filtration on Sephadex G-150

Introduction: Freeman and Smith (1970) report a fractionation of human serum on a Sephadex G-150 column. In their demonstration of over sixty distinct serum proteins, they find only three that are excluded from this gel and are eluted with the void volume. Because most of the serum proteins would seem to have molecular weights of less than 400,000 (the exclusion limit for globular proteins on G-150), Sephadex G-150, superfine, was chosen to attempt further separation of the glycoproteins in Fraction 9 from DEAE-Sephadex (Figure 3).

A 10.0 ml sample from the pooled Fraction 9 was concentrated as described in *Methods*. Fraction 9 contained ^{3}H-labelled glycoproteins from normal and ^{14}C-labelled material from deficient rats. The concentrated sample was applied to a G-150 column, 2.2 x 100 cm, prepared as outlined in *Methods*. The column was run at a constant flow rate of 15 ml per hour and fractions were collected every 10 minutes (2.5 ml per fraction).

Results: The sample placed on the column contained approximately 1.7×10^5 DPM ^{3}H and 1.2×10^5 DPM ^{14}C in 1.0 ml.

Aliquots from each fraction were taken and counted for radioactivity. These counts, corrected for spillover and efficiency, are plotted in Figure 7.

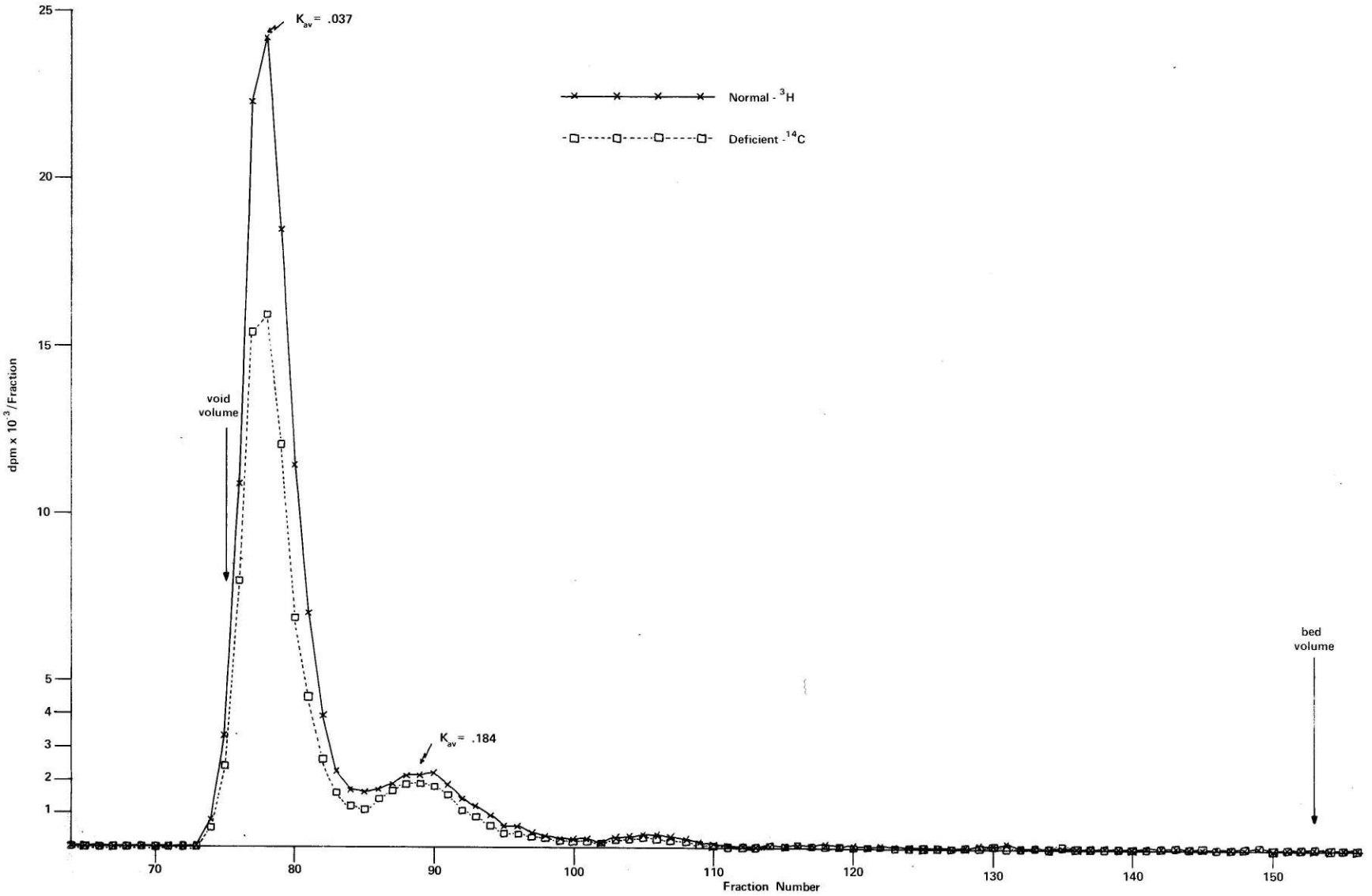
Values for the K_{av} (the partition coefficient between the liquid and the gel phase) were calculated for each peak using the following formula: $K_{av}=V_e-V_o/V_t-V_o$; Where V_o is the void volume; V_t is the bed volume; and V_e is the elution volume of the peak. For these calculations, V_e was determined for the center of each peak. For the G-150 column, V_o was 188 ml, and V_t was 380 ml. K_{av} are included in Figure 7.

The bulk of the labelled material is eluted in fractions 73-83. This peak is very sharp and has a K_{av} of only .037. The ratio of ^{14}C to ^{3}H

FIGURE 7

Fractionation of Pooled Fraction 9

on Sephadex G-150, Superfine


K_{av} = .037
Normal - ^{3}H
Deficient - ^{14}C
void volume
K_{av} = .184
bed volume
dpm x 10^{-3}/Fraction
Fraction Number
25
20
15
10
5
4
3
2
1
70
80
90
100
110
120
130
140
150

(deficient:normal) in this peak is .68 compared with .70 for the sample as applied. From the K_{av} value and the fact that this peak begins rising before the void volume, one can estimate a molecular weight of *at least* 300,000-400,000; if this material is, in fact, excluded from the gel, it could have any molecular weight over 400,000. (All approximate molecular weights reported from gel filtration were arrived at by using curves for globular proteins relating the K_{av} to the molecular weight found in the Sephadex and Sepharose preparation manuals, published by Pharmacia.)

The smaller peak, centered around fraction 89, has a K_{av} of .184. The ^{14}C-^{3}H ratio from fractions 84-94 is .83. Assuming an exclusion limit of 400,000 MW, this peak contains material of about 100,000 MW.

It should be noted that significant losses occurred during the ultrafiltration step used in the preparation of the sample. However, the ratio of the two isotopes in Fraction 9 did not change.

Without drawing any conclusions from this experiment, it was decided to repeat the gel filtration using Sepharose 6B instead of G-150.

C. Fractionation of Pooled Fraction 9: Gel Filtration on Sepharose 6B

Introduction: A 10.0 ml sample from Fraction 9 was prepared as before. A Sepharose 6B column, 2.4 x 45 cm, was poured and equilibrated as described in *Methods*. The flow rate for this run was 36 ml per hour, and fractions were collected every five minutes (3.0 ml per fraction).

Results: The radioactivity applied to the column was 1.77×10^5 DPM ^{3}H and 1.24×10^5 DPM ^{14}C. The sample volume was 2.0 ml.

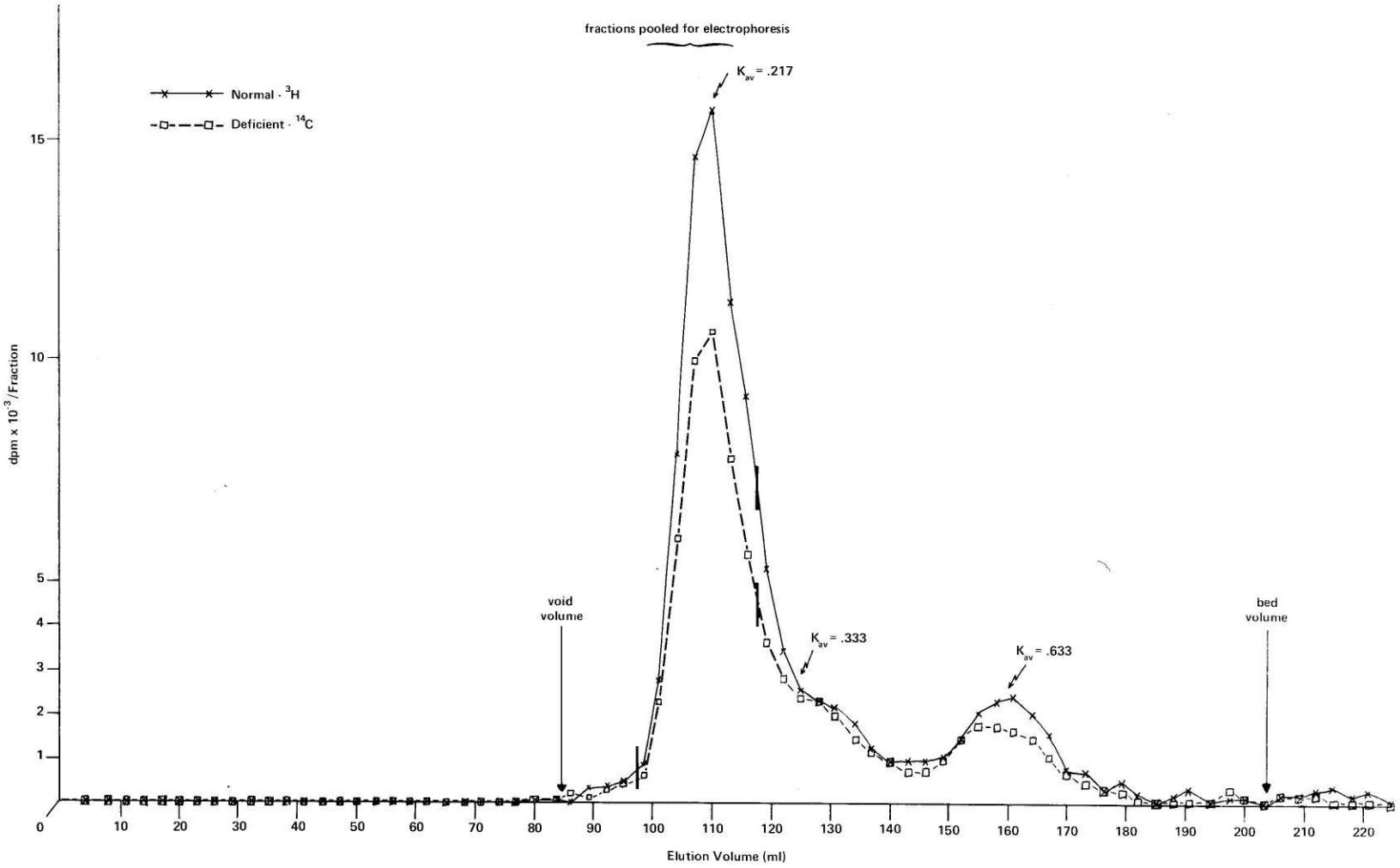
Aliquots were counted and corrected as before and plotted in Figure 8.

For the Sepharose column, V_o was 84 ml, and V_t was 205 ml.

FIGURE 8

Fractionation of Pooled Fraction 9

on Sepharose 6B


fractions pooled for electrophoresis
K_{av} = .217
Normal · ^{3}H
Deficient · ^{14}C
dpm x 10^{-3}/Fraction
15
10
5
4
3
2
1
0
void volume
K_{av} = .333
K_{av} = .633
bed volume
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220
Elution Volume (ml)

Values for K_{av} were calculated for each peak as done previously and included in Figure 8.

The elution pattern obtained here is much like that found in the previous experiment. The major peak (K_{av}=0.217) appears as it did before despite its increased distance from the void volume. The ^{14}C-^{3}H ratio is .71, and its elution volume from start to finish is approximately 30 ml; these figures were .68 and 25 ml for the similar peak off G-150.

The smaller peak with K_{av}=0.633 corresponds well with the K_{av}=0.184 peak from G-150 (Figure 7). Comparing the ^{14}C-^{3}H ratios for this peak again shows good correlation with these being .81 and .83 from Sepharose and G-150 respectively.

On Sepharose, a third peak (not seen on G-150) can be noted, half-buried in the major peak. The center of this peak was estimated to occur at V_e= 125 ml from which a K_{av} of .333 was calculated. The appearance of this peak from Sepharose, but not from G-150, which was run at a much slower flow rate and is a more selective gel, strongly suggests that the major peak seen in the Sephadex G-150 elution profile (Figure 7) does represent material excluded from the gel (molecular weight greater than 400,000).

Molecular weights can be estimated from Sepharose 6B elution results and are: K_{av} = .217, 1 x 10^6 MW; K_{av} = .333, 400,000 MW; and K_{av} = .633, 50,000-100,000 MW.

The fractions making up the major peak from this experiment were pooled to assess its purity by subjecting it to disc electrophoresis. The region pooled is indicated in Figure 8; the sample was stored at 4^{o} C until used. (This pooled peak shall be referred to as "Peak A" from here on.)

C. Fractionation of Pooled Fraction 9: Polyacrylamide Gel Electrophoresis

Introduction: Preparation of sample and gels were carried out as outlined under *Methods* as was protein and carbohydrate staining and gel radioactivity determination. Three gels were run, one for each assay.

Results: Gels stained for protein and carbohydrate are shown in Figure 9. The radioactivity profiles of the third gel are plotted in Figure 10.

The bulk of the material appears in a single band with Rf = .64, .64 and .65 respectively in protein, carbohydrate and radioactivity gels. This band contains more than 90% of the total gel counts and has a ^{14}C-^{3}H (deficient:normal) ratio of .72 (slices 22-30 in Figure 10). It is interesting to note here the same peak shift seen on DEAE-Sephadex; such a shift was not evident in either of the gel filtration experiments. Its reappearance suggests that a charge difference between deficient and normal material is responsible for this shift, the deficient glycoprotein being the more acidic in both systems (more negatively charged). A molecular weight difference cannot be totally ruled out by the absence of this shift effect in gel filtration because of the low selectivity of the gel sizes used; migration is effected by molecular weight in polyacrylamide gels due to a sifting effect.

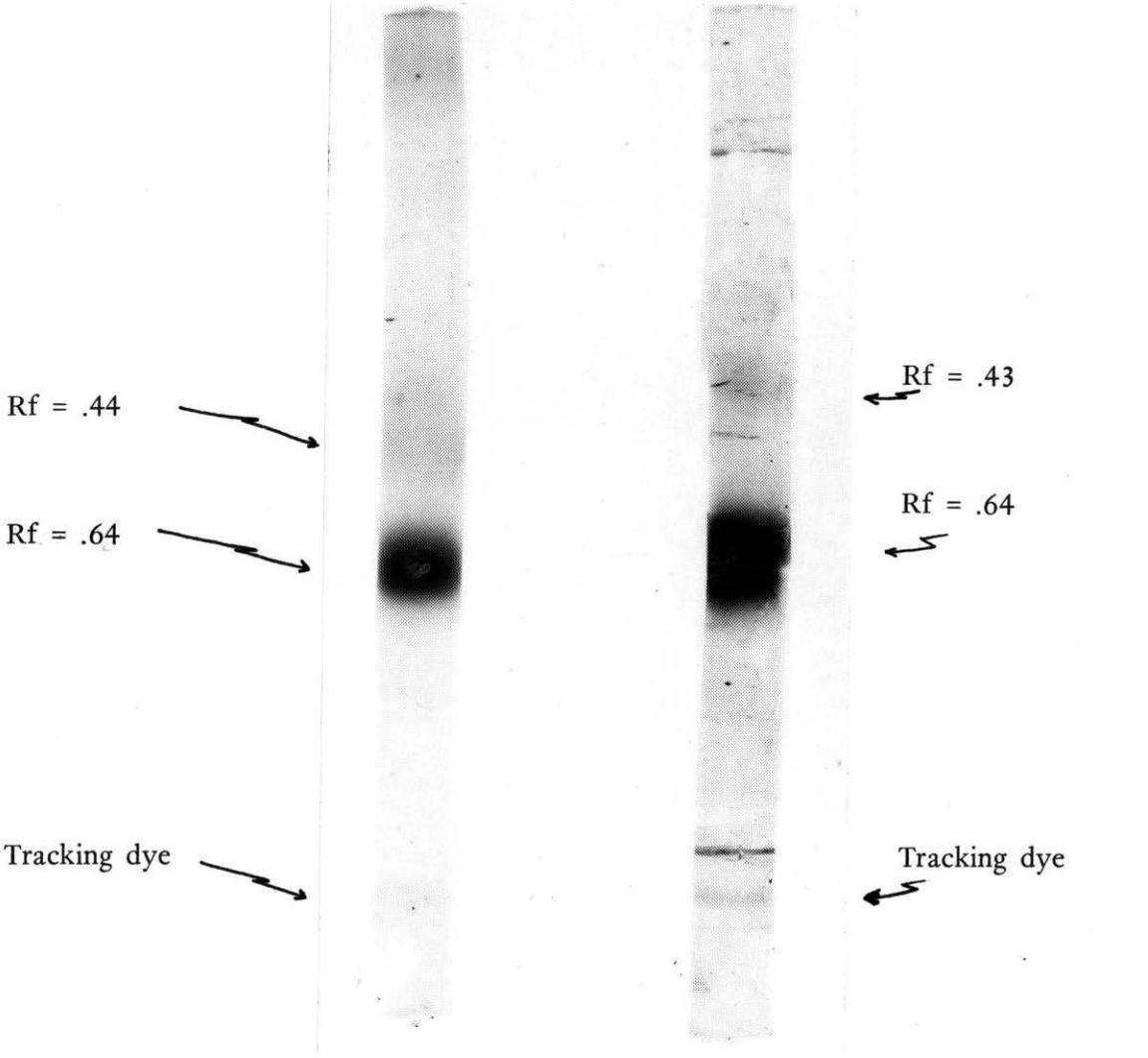
A much smaller peak, probably corresponding to the material in the K_{av} = .333 peak from Sepharose 6B (Figure 8), is also evident in all three gels. Protein, carbohydrate and radioactivity gels give Rfs of .43, .44 and .47 respectively for this band. The ^{14}C-^{3}H ratio is .67 for this band; this radioactivity represents about 5% of the total gel counts.

Low level radioactivity (less than 20.0 DPM per slice) is present in the remainder of the gel. Some faint staining of these is observed with the PAS (carbohydrate) stain, but no corresponding protein was detectable in the gel stained with amido black.

FIGURE 9

5% Polyacrylamide Gel Electrophoresis of Peak A:

Results from Carbohydrate and Protein Staining

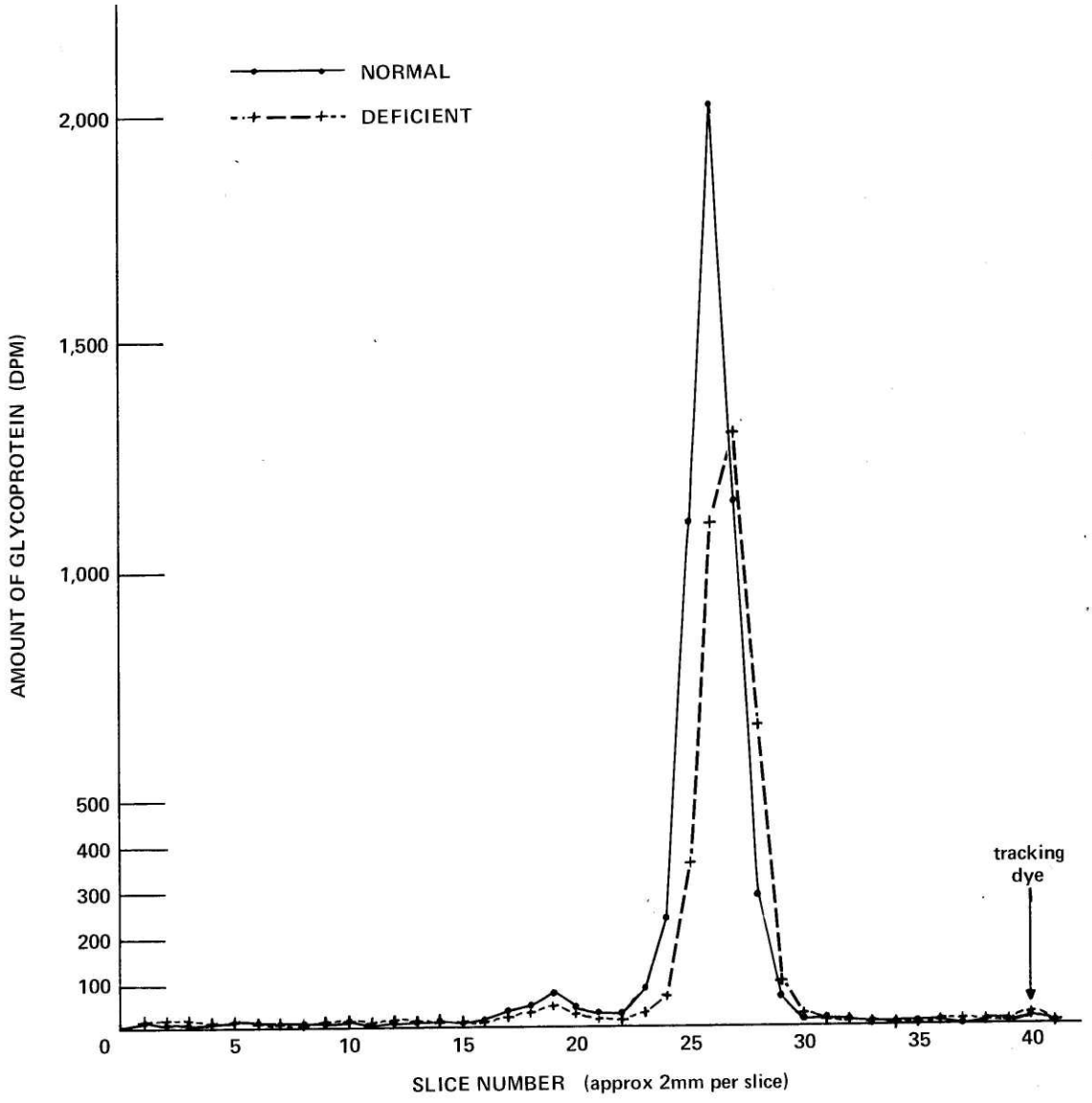


Note: the gel stained for protein was broken while destaining. Marks not indicated are breaks or dirt outside of the gel.

FIGURE 10

5% Polyacrylamide Gel Electrophoresis of Peak A:

Results from Radioactivity Determination after Slicing



Standard mobilities were calculated for the two bands based on the migrations measured on the PAS stained gel. These are: Rf = .64, -1.59 x 10^{-5} cm^2/volt/sec; Rf = .44, -1.09 x 10^{-5} cm^2/volt/sec.

C. Fractionation of Pooled Fraction 9: Discussion

The results of the three preceeding experiments indicate the following:

1. The majority of the radioactivity in the pooled Fraction 9 is associated with a single glycoprotein;

2. This glycoprotein, in the form observed here, has a molecular weight on the order of 1 x 10^6 MW from its behavior on Sepharose 6B;

3. It is negitively charge above pH 8.0 with a mobility of -1.59 x 10^{-5} cm^2/volt/sec in 5% polyacrylamide gel electrophoresis at pH 8.6; its elution on DEAE-Sephadex suggests that this is an $alpha_1$-globulin;

4. As guaged by measurements of its radioactivity, this material shows decreased levels of radioactivity in the plasma of vitamin A-deficient rats when compared to that of pair-fed normals; the rate of incorporation of label into this glycoprotein would appear to be about 30% lower in vitamin A-deficiency than that found in normal rats by this determination.

Two other glycoproteins effectively account for the remaining radioactivity in the pooled Fraction 9. Both are decreased in vitamin A-deficiency. One, with a molecular weight around 400,000, has an approximate mobility of -1.09 x 10^{-5} cm^2/volt/sec in 5% polyacrylamide gel electrophoresis (pH 8.6). The other glycoprotein was not characterized by electrophoresis but has a molecular weight in the range of 50,000-100,000 from its behavior in gel filtration. The radioactivity of this material shows less of a depression in vitamin A-deficiency than the other two, about 20% down from that of the normal rats.

The molecular weights reported for these components are very approximate. Such determinations by gel filtration are crude at best as they are sensitive to factors of flow rate, buffer ionic strength and molecular configuration. However, their inclusion here is merely intended to suggest the order-of-magnitude molecular weight range of the glycoproteins present in the pooled Fraction 9. For this purpose, I feel these approximate figures are of value.

The major component isolated is, almost without question, large in comparison with the majority of the other plasma proteins. Few of these have molecular weights above 200,000, and most are less than 150,000 MW (Winzler and Bocci, 1972). The behavior of this glycoprotein, when subjected to gel filtration, definately places it at a molecular weight above 400,000. Freeman and Smith (1970) found only three serum components in the void volume peak when fractionating human serum on Sephadex G-150. These three were beta-lipoprotein, $alpha_2$ macroglobulin and haptoglobin. All three of these glycoproteins are major constituents of human plasma (Schultze and Heremans, 1970). Among the other significant human plasma glycoproteins, only the alpha-lipoproteins, the immunoglobulins, $beta_{1c}$-globulin and ceruloplasmin have molecular weights of 150,000 or more (Winzler and Bocci, 1972).

Until further work, directed at identifying the affected component, is done, it is impossible to do any more than suggest some of the possibilities. Fortunately, because of its size with relation to the most of the plasma glycoproteins and because of the high level of radioactive precursor incorporated (indicating a high rate of synthesis), positive identification of this component should not be difficult to make.

FINAL DISCUSSION

When rat plasma glycoproteins are labelled by the uptake of radioactive D-glucosamine or D-mannose and fractionated on DEAE-Sephadex A-50, the resulting elution pattern indicates a depression, in vitamin A-deficiency, in the synthesis of several glycoproteins. This apparent decrease in synthesis rate appears when the comparison between vitamin A-deficient and normal rat plasma is based on equal amounts of protein. For one fraction, this effect has been evident in three fractionations with D-glucosamine as the labelled precursor and in one where radioactive D-mannose was the injected sugar. This fraction is believed to contain the $alpha_1$-globulins. Another fraction (believed to be the $alpha_2$-globulins), appears depressed, to a lesser degree, only when glucosamine is the labelling sugar; this material seems to show increased synthesis when mannose is used. Upon further clarification, the $alpha_1$ fraction is found to be dominated, in terms of radioactivity, by a single glycoprotein of high weight (in comparison to the majority of the plasma proteins). This glycoprotein appears to be homogeneous, when subjected to disc electrophoresis in a 5% polyacrylamide gel at pH 8.6.

The effects seen in these studies are believed to be caused by an alteration in the rate of synthesis of particular plasma glycoproteins by the liver in vitamin A-deficiency. Several arguements can be made in support of this hypothesis. First, normal half-lives of plasma glycoproteins are on the order of days (Winzler and Bocci, 1972). As a result, the labelled glycoproteins synthesized by the liver over a period of three hours would not be expected to be catabolized significantly by the end of this time, and therefore any differences seen should reflect changes in the rate of synthesis.

Suppose, though, that catalysis is increased in vitamin A-deficiency. Morell, *et al.* (1971) have suggested that the removal of sialic acid by the plasma neuraminidases initiates the catalysis of plasma glycoproteins. If this is true, then an increase in the catabolism of a particular glycoprotein would imply that the neuraminidase responsible for removing the sialic acid residues of this glycoprotein has an increased activity as well. For my own experiments, if the apparent decrease in radioactivity levels seen for certain glycoproteins in vitamin A-deficiency is due to increased catabolism, the glycoproteins not yet removed from the plasma by the liver after three hours would be expected to have lost more of their sialic acid than the corresponding glycoproteins in normal plasma. This would result in a lower acidity (less negative charge) for the vitamin A-deficient glycoproteins. The shift observed on DEAE-Sephadex and in electrophoresis indicates the exact opposite; it is the deficient glycoproteins which are the most acidic. In the light of this evidence, the likelihood that the observed depressions are due to an increase in the rate of breakdown in vitamin A-deficiency seems very small indeed. There is the possibility that the decreased levels are due to the inhibited secretion of certain glycoproteins in the liver. However, since all glycoproteins are thought to be packaged by the Golgi apparatus and secreted by reverse pinocytosis (Winterburn and Phelps, 1972), such an inhibition of secretion would be expected to affect all glycoproteins made by the liver, not just a few. Since this is not what is observed, control by vitamin A at the level of secretion from the liver does not represent a satisfactory explanation for the results obtained in these studies.

The occurrence of the shift is both interesting and baffling. It represents a general effect in vitamin A-deficiency, the nature of which is a complete mystery to me at this time. Several possible explanations are offered

in the discussion of the DEAE-Sephadex fractionations. If the existence of this basic difference can be more firmly established, its investigation should provide fertile ground for future research.

As for the identify of the glycoprotein found to be the major component of the pooled Fraction 9 by radioactivity measurements, several points should be emphasized. First, it apparent molecular weight puts it well above the mass of the plasma glycoproteins (Winzler and Bocci, 1972). Secondly, the assignment of this glycoprotein to the $alpha_1$-group seems reasonable based on the cellulose acetate electrophoresis of human serum cited previously (Klainer, *et al.*, 1968). Finally, while the radioactivity measurements are not conclusive in this regard, it is almost certain that this glycoprotein exists in the plasma at high levels; certainly it must be a member of the select thirteen glycoproteins known to account for over 90% of the glycoproteins (minus the immunoglobulins) by mass in human serum (Schultze and Heremans, 1970). While all these facts would seem to make the identification of this glycoprotein a simple proposition, very little work has been done in the characterization of plasma glycoproteins in the rat. This is mainly due to the availability of human and bovine plasma in large quantities. As a result, identification will not be simply arrived at by establishing the characteristics and looking up the known glycoprotein in the literature. More likely, comparisons will have to be made with the better-characterized glycoproteins of human plasma, for example, with a resulting loss in certainty.

In returning to the liver, the mode of action of vitamin A, which results in a depression of the synthesis rate of the isolated glycoprotein in vitamin A-deficiency, is not to be found by looking at the effects in the plasma. To be sure, these observations are what one would expect to find if

retinol is functioning in the liver as a sugar carrier linked to the glycosylation of synthesizing glycoproteins, as the work of De Luca, *et al.* (1973) has suggested. However, the effect alone can offer little in answer to the question, "Why?" Extensive work in the future must, and hopefully will, be done to bridge the canyon of unknowns that lies between the effect and the cause.

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