Early effects of feeding excess vitamin A: mechanism of fatty liver production in rats

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ABSTRACT Oral administration of vitamin A (30,000 IU daily for 2 days) to young rats caused a marked increase in hepatic glycogen, cholesterol, and glycerides, while hepatic phospholipid content remained almost unaltered.

In an examination of the pathogenesis of the lipid accumulation, it was found that more glucose-^{14}C was incorporated into liver lipids in vitamin A-fed rats, whereas incorporation of glucose-^{14}C and DL-glycine-^{14}C into liver protein remained unaltered. The increase in glucose-^{14}C incorporation was confined to the glyceride-glycerol portion of the lipids; incorporation into liver fatty acids was inhibited. Plasma free fatty acid concentrations were elevated.

It is postulated that in the vitamin A-fed rats, increased accumulation of lipids in the liver is caused by a stimulation of fatty acid mobilization from adipose tissue and enhanced formation of glycerophosphate through glycolysis, with consequent increase in the glyceride synthesis in the liver.

The weight of the adrenals was increased, whereas cholesterol concentration in the gland was decreased, after administration of vitamin A to rats. This indicates adrenocortical stimulation. Interestingly enough, vitamin A feeding did not affect either the level of liver lipids or of plasma FFA in adrenalectomized rats.

SUPPLEMENTARY KEY WORDS lipid mobilization · glyceride-glycerol synthesis · adrenal weight · cholesterol · ascorbic acid · adrenalectomy

It is now well recognized that ingestion of large doses of vitamin A causes toxic manifestations (1–5). Earlier reports from this laboratory have shown considerable elevation in the lipid content of rat liver as a result of intramuscular administration of 100,000 IU of vitamin A daily for total periods of 10–14 days (6, 7). However, these studies were confined to changes in the levels of various lipid components of the liver, and the mechanism of vitamin A-mediated accumulation of lipids in the liver has not as yet been explained. The present investigation was, therefore, undertaken to study the early effects of feeding excess vitamin A (30,000 IU daily for 2 days) on the lipids of the liver, on the plasma free fatty acids (FFA), and on the lipogenic capacity of the liver of young (60–90 g of body weight) rats. In this study glucose-U-^{14}C has been chosen as a precursor for the study of lipogenesis, because it is the only physiological precursor that is of quantitative significance in lipogenesis. Furthermore, in view of the known effects of adrenal hormones on the mobilization of lipids from adipose tissue (8–10), we were prompted also to study the effects of vitamin A on adrenal cortical function and on liver lipids and plasma FFA of adrenalectomized rats.

Abbreviation: FFA, free fatty acids.

MATERIALS AND METHODS

Male albino rats weighing 60–90 g were divided into two groups, "vitaminotic" and "control." Each animal in the vitaminotic group was fed 30,000 IU of retinol in 0.4 ml of ethyl oleate (Prepalin, Glaxo Ltd., Bombay, India) once daily for 2 days, whereas animals of the control group received appropriate volumes of ethyl oleate. The rats were killed about 24 hr after administration of the second dose. The animals, which had been fasted overnight (18–20 hr), were stunned by a blow on the head and quickly exsanguinated after decapitation. The liver was excised and dropped immediately into chilled (0–4°C) physiological saline solution. The tissue was quickly used for various analyses.

Estimation of Plasma FFA

FFA was determined in freshly obtained plasma by the method of Dole and Meinertz (11).

Estimation of Liver Glycogen

Immediately after the animals had been killed, the right lobe of the liver was quickly excised, cleaned of extraneous materials, and dropped into 30% aqueous KOH. Glycogen was isolated (12), purified by reprecipitating twice with alcohol, and estimated by the phenol–sulfuric acid method (13).

Estimation of Liver Lipids

A portion of the liver (about 1 g) was dropped into 20 volumes of chloroform–methanol 2:1 and finely ground with the help of a glass pestle and mortar. Lipid was then extracted and purified (14). Aliquots of the lipid solution in chloroform were used for the estimation of total lipids (gravimetrically), phospholipid [phosphorus (15, 16) × 25] and cholesterol (17). Glyceride was estimated either by difference (total lipid minus phospholipid and cholesterol) or by determination of glyceride-glycerol (18). In the former case, we corrected for the contribution of vitamin A on the assumption that all the vitamin in the liver (determined as described below) was in the form of retinyl palmitate. Before the measurement of glyceride-glycerol, phospholipids were removed from the neutral lipids as follows. An aliquot of the lipid solution (about 4 mg of lipid) in chloroform was mixed and shaken with 0.3 g of silicic acid (100–200 mesh) (19). The silicic acid was removed by centrifugation at about 2000 *g* for 10 min and washed with 4 × 3 ml of chloroform, each time with centrifugation. The combined solutions were evaporated to dryness and the residue (neutral lipids) was redissolved in a known volume of chloroform for glyceride-glycerol estimation (18), with tripalmitin as a standard.

Aliquots of the lipid solution in chloroform were used for the estimation of vitamin A by the antimony trichloride procedure of Carr and Price as described by Embree, Ames, Lehman, and Harris (20).

Protein Determination

2 volumes of 10% trichloroacetic acid were added to a suitable amount of 10% liver homogenate in 0.25 M sucrose and the insoluble residue was washed with ethanol–ether (3:1) mixture and then with ether. The dry powder thus obtained was dissolved in 0.1 N NaOH and protein content was estimated by the biuret method (21).

Estimation of Deoxyribonucleic Acid

Deoxyribonucleic acid (DNA) was isolated from liver by the method of Schmidt and Thannhauser as described by Munro and Fleck (22). DNA content was determined by measuring deoxyribose.

Incorporation of Glucose-U^{14}C Into Liver Lipids

To rats which had been fasted for 18–20 hr, 30 μc (200 mg) of glucose-U-^{14}C was fed per 100 g of body weight; 3 hr later they were killed. Total lipids were isolated from about 2 g of the liver (14). An aliquot of the lipid solution in chloroform was plated on stainless steel planchets and radioactivity was determined in a gas-flow counter with an efficiency of about 40%. The remaining solution was evaporated to dryness under nitrogen and the lipids were saponified (23). Nonsaponifiable materials and fatty acids were extracted into petroleum ether (boiling range 40–60°C) from the alkaline alcoholic digest (24). The aqueous layer was regarded as the glyceride-glycerol fraction. The validity of this procedure for measuring glyceride-glycerol-^{14}C has been tested and confirmed (25). Suitable aliquots of these fractions were plated in stainless steel planchets for radioactivity determination.

Incorporation of ^{14}C-Labeled Precursors into Liver Proteins

Rats fasted overnight (18–20 hr) were fed either 30 μc (200 mg) of glucose-U-^{14}C/100 g of body weight or intraperitoneally injected with 15 μc (specific activity 5.2 mc/mmole) of DL-glycine-2-^{14}C/100 g of body weight. 3 hr later, animals were killed and the liver was excised and homogenized in chilled (0–4°C) 0.25 M sucrose solution. 2 volumes of 10% trichloroacetic acid was added to an aliquot of the liver homogenate. The trichloroacetic acid-insoluble material was collected by centrifugation and freed from acid-soluble compounds, nucleic acids, and lipids (26). The dry protein was dissolved in a suitable volume of concentrated (85%) formic acid. An aliquot of this solution was plated for radioactivity determination.

Determination of Adrenal Cholesterol and Ascorbic Acid

The adrenal glands were excised, cleaned of adhering fat, and finely ground in chloroform–methanol 2:1. The chloroform–methanol extract was evaporated to dryness and lipids were extracted with chloroform. An aliquot of the chloroform solution was taken for cholesterol determination (17).

The adrenals were finely ground in cold (0–4°C) 5% trichloroacetic acid and ascorbic acid was estimated by the method of Roe and Keuther (27).

Studies on Adrenalectomized Rats

Rats weighing 70–85 g were bilaterally adrenalectomized and were maintained on 0.9% saline solution instead of drinking water throughout the experimental period. 5 days after adrenalectomy, rats were divided into two groups, "control" and "vitaminotic." Each vitaminotic rat was fed 30,000 IU of vitamin A in ethyl oleate once daily for 2 days, whereas control animals were admin

istered appropriate amounts of the ethyl oleate for the same period. Liver lipids and plasma FFA were then determined as described above.

RESULTS

It is evident from the data presented in Table 1 that administration of excess vitamin A for only 2 days increased liver weight and hepatic glycogen, vitamin A, and lipid contents, but led to no change in the protein level. The decrease in hepatic DNA level was not statistically significant. In subsequent studies we investigated the effect of feeding excess vitamin A on the concentration of the various major components of liver lipids. The findings are given in Table 2. Results have been expressed as mg/g of liver and also as mg/mg of hepatic DNA. The latter parameter has been used to assess lipid content per cell. Hepatic cholesterol and glycerides per mg of DNA increased by 90 and 100%, respectively, as a result of feeding vitamin A. The phospholipid content remained essentially unaltered.

Plasma FFA were estimated as an indicator of the effect of vitamin A on the lipid mobilization from the adipose tissue. Table 3 shows that vitamin A administration caused more than an 85% elevation in the concentration of plasma FFA. 3 hr after feeding 2 g of glucose/kg of body weight, plasma FFA concentration in the control animals was reduced by about 25%, whereas that of the vitaminotic rats did not change.

To assess the lipogenic capacity of the liver, we studied the in vivo incorporation of glucose-U-^{14}C into lipids (Table 4). Vitamin A feeding increased the ^{14}C-incorporation into total lipids, the increase being about 40% when the results were expressed as cpm/mg of DNA. The lipids were saponified, and nonsaponifiable compounds, fatty acids, and glyceride-glycerol were separated. The ^{14}C count of each fraction showed that the observed increase in the incorporation of glucose-^{14}C into lipids was confined to the glyceride-glycerol moiety of the lipid. The increase in ^{14}C-incorporation into the glyceride-glycerol was more than 50%, expressed as cpm/mg of DNA. In contrast, incorporation of glucose-^{14}C into fatty acids of the lipids was significantly lowered. ^{14}C-incorporation into the nonsaponifiable lipid fraction was unaffected.

To see the effect of feeding vitamin A on the ability of the liver to synthesize protein, we investigated the incorporation in vivo of glucose-U-^{14}C and glycine-2-^{14}C into liver protein. As can be seen from Table 5, incorporation of both glycine-^{14}C and glucose-^{14}C into liver protein was unaffected by vitamin A feeding.

The adrenal weight and adrenal cholesterol and ascorbic acid contents were determined in vitamin A-fed rats, in an effort to learn the effect of vitamin A on adrenocortical function. The administration of the vita-

TABLE 1 EFFECT OF FEEDING EXCESS VITAMIN A ON WEIGHT AND MAJOR CHEMICAL CONSTITUENTS OF RAT LIVER

	Control	Vitaminotic	*P*
Liver wt (g)	2.79 ± 0.08 (38)	3.25 ± 0.16 (38)	<0.001
Liver/100 g of body wt (g)	3.36 ± 0.12 (38)	4.10 ± 0.19 (38)	<0.005
Protein (g/100 g of liver)	21.0 ± 0.31 (10)	20.5 ± 0.48 (10)	
Total lipid (mg/g of tissue)	38.9 ± 1.78 (6)	50.7 ± 3.46 (6)	<0.02
Glycogen (g/100 g of liver)	0.22 ± 0.02 (18)	2.90 ± 0.23 (18)	<0.001
DNA (mg/g of liver)	2.64 ± 0.15 (6)	2.36 ± 0.13 (6)	
Vitamin A (μg/g of liver)	22.80 ± 3.10 (6)	1163 ± 96 (6)	<0.001

Each value given in the Table is mean ± SEM. The number of animals is given in the parentheses.

TABLE 2 EFFECT OF FEEDING EXCESS VITAMIN A ON LIPIDS OF RAT LIVER

Lipid Fraction	per g of Liver		per mg of DNA	
	Control	Vitaminotic	Control	Vitaminotic
Total lipid (mg)	38.9 ± 1.78	50.7 ± 3.46 $P < 0.02$	14.8 ± 0.67	21.8 ± 1.48 $P < 0.005$
Phospholipid (mg)	23.7 ± 1.50	23.2 ± 1.24	9.00 ± 0.57	10.0 ± 0.52
Cholesterol (mg)	2.20 ± 0.12	3.90 ± 0.22 $P < 0.001$	0.83 ± 0.05	1.67 ± 0.08 $P < 0.001$
Glycerides* (mg)	12.8 ± 1.09	21.4 ± 2.82 $P < 0.02$	4.82 ± 0.40	9.15 ± 1.08 $P < 0.005$
Glyceride-glycerol (μmoles)	14.4 ± 0.85	23.4 ± 1.28 $P < 0.005$	5.53 ± 0.39	9.91 ± 0.62 $P < 0.001$

Each value is mean ± SEM from six separate experiments (six animals).
* Obtained by subtracting phospholipid plus cholesterol from the total lipids. In the case of vitamin A-fed rats, correction was made for the presence of vitamin A, on the assumption that all the vitamin A present in the liver existed as retinyl palmitate.

TABLE 3 LEVELS OF PLASMA FFA IN CONTROL AND VITAMIN A-FED RATS

Nutritional State	Plasma FFA		P
	Control	Vitaminotic	
	μeq/liter		
Fasted	712 ± 33.0	1330 ± 30.0	<0.001
Glucose-fed*	527 ± 22.2	1300 ± 69.4	<0.001

* Animals were fed glucose (2 g/kg of body wt) 3 hr before they were killed.

Each value given in the Table is mean ± SEM from six separate experiments (six animals).

min resulted in considerable increase in the weight and marked decrease in the cholesterol content of the adrenal glands (Table 6). The decrease in ascorbic acid content was not statistically significant.

The effects of vitamin A on the liver lipids and plasma FFA of adrenalectomized rats were investigated. As evident from Table 7, in the absence of intact adrenals, vitamin A did not affect either the level of the liver lipids or that of plasma FFA.

DISCUSSION

The results show that feeding excess vitamin A (30,000 IU daily) for even as short a period as 2 days causes increased accumulation of lipids in rat liver (Table 1). The increase was only in the glyceride and cholesterol fractions of the lipid the phospholipid content remaining unaltered (Table 2). The knowledge gained from the studies of fatty livers caused by the administration of ethionine, CCl_4, puromycin, and ethanol (28–31) has led to the concept that the development of fatty liver is due to one (or a combination of more than one) of the following factors: increased hepatic lipogenesis, enhanced mobilization of FFA from the adipose tissue, decreased oxidation of fatty acids in the liver, or impairment of the hepatic triglyceride secretory mechanism.

In the present study, vitamin A administration caused marked lowering of the glucose-^{14}C incorporation into the liver fatty acids (Table 4). Enhanced hepatic synthesis of fatty acids as a factor causing accumulation of hepatic glycerides in vitamin A-fed rats can therefore be ruled out.

Plasma FFA levels were estimated and used as a measure of lipid mobilization from the adipose tissue. There was a more than 85% increase in plasma FFA level as a result of feeding excess amounts of vitamin A (Table 3). Increased mobilization of fatty acids from the adipose tissue may thus be considered a potentially major factor responsible for the excessive lipid accumulation in the vitamin A-fed rats.

It is now well recognized that triglycerides are synthesized in the liver from fatty acids and glycerol 3-phos-

TABLE 4 INCORPORATION OF GLUCOSE-U-^{14}C INTO LIPID FRACTIONS OF THE LIVER OF CONTROL AND VITAMIN A-FED RATS

Lipid Fraction	cpm/g of Liver		cpm/mg of DNA	
	Control	Vitaminotic	Control	Vitaminotic
Total lipid	11,500 ± 964	14,600 ± 1,870 $P < 0.2$	4,300 ± 231	6,110 ± 570 $P < 0.025$
Fatty acids	1,390 ± 271	702 ± 78 $P < 0.05$	515 ± 60	311 ± 25 $P < 0.01$
Cholesterol	660 ± 194	618 ± 35	240 ± 70	258 ± 16
Glyceride-glycerol	9,600 ± 512	13,300 ± 660 $P < 0.005$	3,610 ± 285	5,510 ± 277 $P < 0.001$

Mean ± SEM is given for data from six separate experiments (six animals).

TABLE 5 EFFECT OF FEEDING EXCESS VITAMIN A ON IN VIVO INCORPORATION OF ^{14}C-LABELED PRECURSORS INTO RAT LIVER PROTEIN

Mode of Expression of Results	DL-Glycine-2-^{14}C		D-Glucose-U-^{14}C	
	Control	Vitaminotic	Control	Vitaminotic
cpm/mg of Protein	306 ± 21.0	328 ± 6.1	75.0 ± 5.1	71.0 ± 4.6
cpm/g of Liver	53,700 ± 1270	50,100 ± 1200	17,200 ± 721	17,500 ± 473
cpm/100 g of body wt*	166,000 ± 3800	17,600 ± 3600	63,200 ± 1970	69,200 ± 2870
cpm/mg of DNA	20,600 ± 402	20,900 ± 348	6,870 ± 348	7,600 ± 373

Mean ± SEM is given for six separate experiments (six animals).

* $\frac{\text{cpm/g of liver} \times \text{total weight of the liver (g)} \times 100}{\text{body wt (g)}}$.

TABLE 6 EFFECT OF FEEDING EXCESS VITAMIN A ON WEIGHT AND CHOLESTEROL AND ASCORBIC ACID LEVELS OF ADRENALS

Measurement	Control	Vitaminotic	P
Total weight (mg)	24.20 ± 0.54 (8)	31.50 ± 0.58 (8)	<0.001
Cholesterol (μg/mg)	17.01 ± 1.66 (8)	8.48 ± 0.72 (8)	<0.001
Ascorbic acid (μg/mg)	3.72 ± 0.55 (4)	2.43 ± 0.37 (4)	<0.1

Each value is mean ± SEM. Number of animals is given in parentheses.

TABLE 7 EFFECT OF FEEDING EXCESS VITAMIN A ON LIVER LIPIDS AND PLASMA FFA OF ADRENALECTOMIZED RATS

Lipid Fraction	Control	Vitaminotic
Total lipid (mg/g of liver)	42.3 ± 2.55	43.5 ± 2.00
Cholesterol (mg/g of liver)	3.72 ± 0.03	3.60 ± 0.14
Phospholipid (mg/g of liver)	21.5 ± 0.89	20.0 ± 0.25
Vitamin A (μg/g of liver)	21.0 ± 1.31	1150 ± 114
Glycerides* (mg/g of liver)	17.1 ± 1.51	17.90 ± 1.70
Free fatty acids (μeq/liter of plasma)	492 ± 43	400 ± 15

Each value is mean ± SEM from five animals.
* Obtained by subtracting cholesterol plus phospholipid from the total lipids. In the case of vitamin A-fed rats a correction for the presence of vitamin A was made, assuming that all the vitamin A present in the liver was in the form of retinyl palmitate.

phate (32). Furthermore, there is ample evidence to show that the rate of triglyceride synthesis in the liver is directly proportional to the concentrations of both the substrates (8, 33–36). As mentioned earlier, the increase in the plasma FFA level was perhaps sufficient to supply fatty acids for enhanced hepatic triglyceride formation in the vitamin A-fed rats. However, for the esterification of fatty acids to bring about increased glyceride synthesis, generation of glycerol phosphate must be simultaneously stimulated. Glycerol phosphate can be formed in liver by two pathways: (*a*) by the action of glycerol phosphate dehydrogenase (EC 1.1.99.5) on the dihydroxyacetone-phosphate formed during the glycolytic breakdown of sugar or during the gluconeogenic process (37), and (*b*) by the phosphorylation of glycerol by the glycerokinase reaction (38). However, of the two pathways, only the first is considered to be of quantitative importance (37). We therefore determined the incorporation of glucose-^{14}C into the glyceride-glycerol in order to assess the production of glycerol phosphate via the glycolytic pathway. It was considerably enhanced (Table 4) in vitamin A-fed rats. Thus, increased synthesis of glycerides as a result of (*a*) enhanced mobilization of fatty acids from adipose tissue and (*b*) increased glycerol phosphate formation via the glycolytic pathway in the liver seems to be a major, if not the only, factor responsible for the fatty liver.

From the present data the effect of vitamin A administration on fatty acid oxidation in the liver cannot be ascertained. However, unlike the fatty liver caused by administration of ethionine, puromycin, or CCl_4 (28, 29), the lipid accumulation in the liver of vitamin A-fed rats cannot possibly be ascribed to diminished lipoprotein formation and (or) secretion into the blood for the following reasons: (*a*) The liver is the major, if not the only, site of synthesis of plasma triglycerides, and the triglycerides formed in the liver are released into the blood in the form of very low density lipoproteins (29, 39). Thus, a diminution of lipoprotein synthesis and (or) secretion would be expected to result in lowering the blood lipids, especially the plasma triglyceride content, as has been reported to occur after the administration of ethionine, CCl_4, puromycin, phosphorus, or orotic acid (28, 29). In contrast, the vitamin A-fed rats show a considerable increase in plasma total lipids and plasma triglycerides (40). (*b*) Since the protein moiety of the very low density lipoproteins is synthesized mainly in the liver (28, 39), a block in the synthesis and (or) secretion of lipoproteins is ordinarily accompanied by a diminished synthesis of protein in the liver (28, 29), except in the case of orotic acid feeding which is known to interfere with the synthesis and (or) secretion of very low density lipoproteins without affecting hepatic protein synthesis (41–46). The data presented in this report show that administration of vitamin A did not affect protein synthesis in the liver. Thus, findings showing elevation in the level of plasma triglyceride (40) and unaltered hepatic protein synthesis provide strong evidence that synthesis and (or) secretion of lipoproteins is probably not impaired in vitamin A-fed rats.

The present findings have brought to light another noteworthy phenomenon, namely, inhibition of fatty acid synthesis from glucose in vitamin A-fed rats (Table 4). It must be pointed out here that, since the acetyl CoA pool was not measured in the present study, the extent to which a possible alteration in the acetyl CoA pool could have influenced the incorporation of ^{14}C from glucose-^{14}C into fatty acids cannot be evaluated. However, in view of the fact that incorporation of glucose-^{14}C into cholesterol and protein was not inhibited by vitamin A feeding, it seems unlikely that the entire decrease of about 40% in the glucose-^{14}C incorporation into fatty acids could have been caused only by a change in the acetyl CoA pool. Furthermore, since incorporation of glucose-^{14}C into cholesterol was not influenced by vitamin A feeding, a block in fatty acid synthesis should be

localized at some step or steps subsequent to acetyl CoA formation (either acetyl CoA carboxylase reaction or subsequent condensation of acetyl CoA and malonyl CoA). However, since acetyl CoA carboxylase reaction has been reported to be a rate-limiting step in the fatty acid biosynthesis (47), a block at this step is conceivable.

Since it has been reported that fatty acids and fatty acyl CoA inhibit the biosynthesis of fatty acids (47), the vitamin A-mediated inhibition of hepatic fatty acid synthesis from glucose-^{14}C could, perhaps, be attributed to increased availability of fatty acids as a result of the enhanced mobilization of lipids from adipose tissue indicated by elevated plasma FFA. Nevertheless, other possibilities such as decreased availability of NADPH cannot be ruled out.

The adrenal weight and its cholesterol and ascorbic acid contents have generally been used as a measure of adrenocortical function. The stimulation of adrenocortical function is ordinarily accompanied by an increase in the weight and lowering of cholesterol and ascorbic acid contents of the gland (48). Thus, the present results (Table 6) can be considered to suggest that vitamin A stimulates the adrenocortical function. It is interesting to note that vitamin A administration to the adrenalectomized rats did not alter either the levels of liver lipids or those of plasma FFA. These findings indicate that intact adrenals are needed for the manifestation of the vitamin A-mediated alterations in the levels of plasma FFA and liver lipids. However, from the present findings it is not clear whether adrenal plays a permissive or a direct role. Also, since complete adrenals (both medulla and cortex) were removed during the adrenalectomy, it is not possible to define the exact role of adrenal medulla and cortex in the vitamin A-mediated changes in the lipid metabolism. It seems pertinent to recall here that adrenalectomy has been reported to abolish or lower the susceptibility to alcohol (30) and ethionine (29) with regard to the development of fatty livers. Furthermore, Wool and Goldstein (49) and Wool, Goldstein, Ranney, and Levine (50) presented evidence to show that both adrenal medulla and cortex were involved in the development of ethionine-mediated fatty liver and suggested that the major role of epinephrine was to mobilize FFA from the adipose tissue and that cortisone perhaps plays a permissive role. The above findings have subsequently been confirmed by Hutterer (51).

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