Secretion of Hepatic Triglycerides into Plasma of Rats Fed Retinol

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The effect of feeding 33 mg of retinol daily for two days on liver and plasma lipids of rats has been studied. The secretion of liver TG into plasma of retinol fed rats has been measured by the use of palmitic acid-1-¹⁴C and of Triton WR 1339. Liver and plasma lipids, TG, phospholipids and PC were significantly higher in retinol fed rats as compared to control rats. The incorporation of palmitic acid-1-¹⁴C into liver TG, PC and PE was significantly higher in retinol fed rats. The labelling pattern with time of liver and plasma TG and PC shows that the secretion of liver TG and PC into plasma was impaired in retinol fed rats.

Oral or intramuscular administration of large amounts of vitamin A to experimental animals causes several characteristic abnormalities.¹⁾ One of the more pronounced effect of hyper-*vitaminosis A* is a large increase in liver triglycerides accompanied by hypertriglyceridemia and hyperphospholipidemia.^{2~5)} Since no studies have been done to explain the mechanism of these effects the present study was undertaken to study the synthesis of TG in liver and their secretion into plasma by the use of palmitic acid-1-¹⁴C and of Triton WR 1339. Several factors have been suggested to be involved in the increased accumulation of triglycerides (TG) in liver observed in several experimental conditions.⁶⁾ These include (i) enhanced synthesis of TG in liver and their normal or impaired secretion into plasma as lipoproteins (ii) normal synthesis of TG in liver and their impaired secretion into plasma. The secretory rate of hepatic TG into plasma of fed rats with a normal fatty liver has been assessed by the use of labelled palmitate and Triton WR 1339.⁷⁾ The results of the present investigation show that the release of hepatic TG into plasma of rats fed excess retinol was impaired.

MATERIALS AND METHODS

Animals and feeding of retinol. Male rats of the

Wistar strain, from the colony maintained in our Institute and weighing 90~100 g were used. The animals were divided in two groups each of 12 rats. They were housed in individual cages and were pair fed a stock diet of the following composition: casein 20%, potato starch 70%, Dalda (a hydrogenated vegetable oil) 10%. In addition vitamin mixture, salt mixture and choline chloride at 2.5, 5 and 1.5% levels respectively were added to the diet.⁸⁾ The animals had free access to water at all times. To one group of rats 33 mg of retinol (La Roche, Bombay) in groundnut oil was given orally daily for two days. The second group received only the vehicle solution and was treated as the control.

Administration of palmitate-1-¹⁴C and triton WR1339. Twenty four hours after administering the last dose of retinol, rats in both groups were injected palmitic acid-1-¹⁴C (10 μ Ci/100 g body weight, specific activity 5.0 mCi/mmol obtained from Bhabha Atomic Research Centre, Bombay) as an albumin complex,⁹⁾ through the tail vein under light ether anaesthesia. A 20% solution of triton WR 1339 (polymeric *p*-isooctyl polyoxy-ethylenephenol) (Winthrop Laboratories, New York, U.S.A.) in 0.9% NaCl solution (50 mg/rat) was simultaneously injected through the tail vein.

Collection of tissue samples. Three rats in both groups were sacrificed 10, 20, 40 and 60 min after the injection of the isotope and of Triton WR 1339. Before sacrifice, blood was collected from the jugular vein into heparinized tubes. The animals were quickly cut open, liver removed, dipped in ice-cold saline, wiped and weighed. Blood was centrifuged for 10 min at 2000 rpm and plasma decanted off.

Analytical procedures. Total lipid was extracted from measured aliquots of plasma and liver with chloroform, methanol (2:1, v/v).⁴⁾ Final solution of lipids was made in a known volume of chloroform and a drop of 0.1 percent butylated hydroxy toluene (Nutritional Biochemicals, Cleaveland, Ohio, U.S.A.) in chloroform, added and stored at -20°C until used for analyses. Triglycerides,¹⁰⁾ phosphatidyl choline and phosphatidyl ethanolamine¹¹⁾ were separated by TLC on silica gel G coated plates. The lipid spots were visualized by a brief exposure to I_2 vapors. Gel areas corresponding in R_f values to standard tripalmitin, phosphatidyl choline (PC) and phosphatidylethanol amine (PE) (Sigma Chemicals, St. Louis, Mo., U.S.A.), co-chromatographed with each run, were scraped into tubes. Triglycerides were eluted from the gel with hexane; ether (1:1, v/v), evaporated to dryness and dissolved in chloroform. One aliquot of the eluates was used for triglyceride estimation¹²⁾ and another aliquot for radioactivity counting. The samples were evaporated to dryness under a stream of air and 15 ml of a scintillation mixture¹³⁾ was added. Radioactivity was measured in a Packard scintillation Counter. Phospholipids were quantitated by direct digestion of the gel.¹⁴⁾ For radioactivity determination the phospholipids in the gel were directly transferred into scintillation vials, and 10 ml of scintillation fluid¹⁵⁾ added. Aliquots of the palmitate-albumin complex were also extracted and counted. Plasma free fatty acids were estimated by the method of Dole and Meinertz,³⁹⁾ using palmitic acid as the standard.

The results were calculated and expressed as follows (a) specific activity, counts/min/mg triglycerides, phosphatidyl choline, phosphatidyl ethanolamine, (b) total activities. Liver: $(\text{cpm/mg}) \times (\text{mg/liver}) \times (100/\text{body-}$

$\text{weight} \times 100/\text{cpm injected}$); plasma: $(\text{cpm/mg}) \times (\text{mg/rat}) \times (100 \times \text{cpm injected})$, using a plasma volume of $5.46 \text{ ml}/100 \text{ g body weight}^{7)}$ (c) liver: lipids $\text{mg}/100 \text{ g body weight}$; plasma: lipids $\text{mg}/100 \text{ ml}$. Results were statistically analysed and differences between the control and retinol fed rats were checked by students 't' test and regarded as significant if $P < 0.05$.

RESULTS

The effect of feeding 33 mg of retinol daily for two days on liver TG, PC and PE is shown in Table I. There was a significant increase in liver TG and PC of retinol fed rats as compared to control (Table I). Liver PE contents were not significantly affected in rats fed retinol. Both plasma TG and PC were significantly high in rats fed retinol as compared to control (Fig. 1).

FIG. 1. Effect of Feeding 33 mg of Retinol Daily for Two Days on Plasma Triglycerides and Phosphatidyl Choline Content of Rats.

Values are mean of 12 animals in each group.

TABLE I. EFFECT OF FEEDING 33 mg RETINOL DAILY FOR TWO DAYS ON BODY WEIGHT, LIVER WEIGHT AND LIVER LIPIDS OF RATS

Values are mean $\pm$ S.E. from 12 animals in each group. Values significantly different from control are indicated by * $p < 0.05$.

Body weight (g)	Control	Retinol fed
Initial	94	95
Terminal	91	86
Liver weight (g)	3.28	4.26
per 100 g body weight	3.81	4.95*
Liver TG (mg/100 g body weight)	9.76 ± 3.17	$21.41 \pm 2.14^*$
Liver PC (mg/100 g body weight)	46.65 ± 3.17	$58.65 \pm 2.35^*$
Liver PE (mg/100 g body weight)	29.05 ± 1.9	33.80 ± 1.90
Plasma free fatty acids (μ Eq/lit plasma)	749 ± 42	$1405 \pm 38^*$

Incorporation of palmitic acid- $1\text{-}^{14}\text{C}$ into liver and plasma TG

Incorporation of palmitic acid- $1\text{-}^{14}\text{C}$ into liver total lipids of control and retinol fed rats at 10, 20, 40 and 60 min after the injection of the isotope and of Triton is shown in Fig. 2. The incorporation of palmitic acid- $1\text{-}^{14}\text{C}$ into total lipids was significantly higher in retinol fed rats at all periods of study as compared to control. The maximum incorporation of the isotope into liver lipids was at 10 min period. The rise and decline of the radioactivity of

liver lipids of control was very rapid. However in the retinol fed rats the decline of the radioactivity was slower (Fig. 2). Incorporation of

FIG. 2. Palmitic Acid-1-¹⁴C Incorporation into Liver Lipids (total activity = (cpm/mg lipid) × (mg/liver) × 100/body weight × 100/cpm injected).

FIG. 3. Palmitic Acid-1-¹⁴C Incorporation into Liver Triglycerides (total activity = (cpm/mg triglycerides) × (mg/liver) × 100/body weight × 100/cpm injected).

FIG. 4. Palmitic Acid-1-¹⁴C Incorporation into Liver Triglycerides (specific activity = counts/min/mg triglycerides of rats orally fed 33 mg of retinol daily for two days. Values are mean of 3 animals in each group.

palmitic acid-1-¹⁴C into liver TG of control and retinol fed rats is shown in Figs. 3 and 4. Total activity (percent injected dose) of liver TG of retinol fed rats, which was maximum at 10 min period was significantly higher than that of control (Fig. 3). However the specific activities (counts/min/mg) of liver TG in retinol fed and control rats were not significantly different (Fig. 4) suggesting that the higher total activity of liver TG observed in retinol fed rats (Fig. 3) might have resulted from retention of the newly incorporated label into TG of these rats.

The palmitic acid-1-¹⁴C radioactivity (percent injected dose) and specific activity (counts/min/mg) appearing in plasma TG of both reti-

FIG. 5. Incorporation of Palmitic Acid-1-¹⁴C into Plasma Triglycerides (total activity = (cpm/mg triglycerides) × (mg/rat) × 100/cpm injected).

FIG. 6. Palmitic Acid-1-¹⁴C Incorporation into Plasma Triglycerides (specific activity = counts/min/mg triglycerides).

nol fed and control rats is shown in Figs. 5 and 6. The accumulation of radioactivity into plasma TG which reached maximum at 40 min was significantly higher in retinol fed rats as compared to control. In tritonized control rats (Fig. 1) plasma TG contents were almost doubled at 60 min after its administration whereas in retinol fed rats, no such increase was observed, but the plasma TG values in retinol fed rats remained higher than control at all periods (Fig. 1).

Incorporation of palmitic acid-1-¹⁴C into liver and plasma PC and PE

Labelling pattern with time of liver PC and PE of control and retinol fed rats is shown in Figs. 7~10. Maximum incorporation of the radioactivity occurred at 10~20 min in both

FIG. 7. Palmitic Acid-1-¹⁴C Incorporation into Liver Phosphatidyl Choline (total activity = $(\text{cpm}/\text{mg PC}) \times (\text{mg}/\text{liver}) \times (100/\text{body weight}) \times 100/\text{cpm injected}$).

FIG. 8. Palmitic Acid-1-¹⁴C Incorporation into Liver Phosphatidyl Choline (specific activity = $\text{cpm}/\text{mg phosphatidyl choline}$) of rats orally fed 33 mg of retinol daily for two days. Values are mean of 3 animals in each group.

PC and PE fraction but the accumulation of radioactivity (percent injected dose) in PC and PE of retinol fed rats was more as compared to control (Figs. 7 and 9). The loss of radio-

FIG. 9. Incorporation of Palmitic Acid-1-¹⁴C into Liver Phosphatidyl Ethanolamine (total activity = $(\text{cpm}/\text{mg PE}) \times (\text{mg}/\text{liver}) \times 100/\text{body weight} \times 100/\text{cpm injected}$).

FIG. 10. Palmitic Acid-1-¹⁴C Incorporation into Liver Phosphatidyl Ethanolamine (Specific activity = $\text{counts}/\text{min}/\text{mg phosphatidyl ethanolamine}$).

FIG. 11. Palmitic Acid-1-¹⁴C Incorporation into Plasma Phosphatidyl Choline (total activity = $(\text{cpm}/\text{mg PC}) \times (\text{mg}/\text{rat}) \times 100/\text{cpm injected}$).

FIG. 12. Palmitic Acid-1-¹⁴C Incorporation into Plasma Phosphatidyl Choline (specific activity = counts/min/mg phosphatidyl choline) of rats orally fed 33mg of retinol for two days. Values are mean of 3 animals in each group.

activity was faster in PC than in PE of retinol fed rats as compared to control, the amount of palmitic acid-1-¹⁴C labelled PC (percent injected dose) appearing in plasma was significantly higher in retinol fed rats as compared to control (Fig. 11). In retinol fed rats the maximum rise in PC radioactivity was at 20min and after that it plateaued off whereas in control rats this rise was slow and gradual, reaching the values of retinol fed rats at 60 min after the injection of the isotope (Fig. 12).

DISCUSSION

Administration of retinol either orally or intramuscularly for varying periods to rats fed *ad libitum* has been reported to increase liver triglycerides and cause hypertriglyceridemia and hyperphospholipidemia.³⁻⁵ Increase in plasma triglycerides and phospholipids has also been reported in humans given large quantity of retinol.^{16,17} The results of the present investigation confirm that oral administration of 33 mg retinol daily for two days causes an accumulation of triglycerides and phosphatidyl choline in liver and plasma as compared to the controls (Table I and Fig. 1). Several conditions of triglyceride metabolism in liver can lead to their accumulation under a variety of experimental manipulations.⁶ Some of these include (a) an enhanced synthesis of triglyceride and their normal or impaired secretion to plasma and (b) normal synthesis of trigly-

cerides and their impaired secretion into plasma. In the reported experiments the above two aspects of triglyceride metabolism have been studied in rats fed retinol. The incorporation of palmitate-1-¹⁴C into liver total lipids triglycerides (Fig. 2 and Fig. 3) of retinol fed rats was significantly higher at all periods studied, after the injection of the isotope as compared to control. However the specific activities (counts/min/mg TG) of TG in control and retinol fed rats were nearly comparable at all periods of study, suggesting that the rate of the synthesis of TG in the two groups was not different (Fig. 4). However if we consider the enlarged pool size of liver TG in retinol fed rats (Table I), then it is noted that inspite of the dilution of the label by the TG pool of retinol fed rats, the specific activity (counts/min/mg TG) of TG was comparable to the controls, showing that the rate of synthesis of TG in retinol fed rats was greater than the controls (Fig. 4). The loss of label from liver TG would indicate its utilization. The results presented in Fig. 3 point out that though the utilization of newly synthesized TG in control and retinol fed rats was nearly comparable, still the TG radioactivity retained in liver of retinol fed rats was higher than the controls. The results suggest that a increased synthesis and an impaired secretion of liver TG in retinol fed rats lead to their increased accumulation as compared to the controls. An impairment in the release of hepatic TG is usually reflected by lower concentration of plasma TG.¹⁸ To assess the ability of liver to secrete TG into plasma in a variety of experimental conditions use of Triton WR 1339, a nonionic detergent has been made.¹⁹⁻²² To circumvent the many limitations of the use of this test in fed rats, Lombardi *et al.* performed this test to estimate liver TG secretion in choline deficient rats by simultaneously injecting labelled palmitic acid and Triton-WR 1339.⁷ To assess the ability of liver to secrete TG into plasma of retinol fed rats, both palmitic acid-1-¹⁴C and Triton-WR 1339 were simultaneously injected and increment in the radioactivity of plasma TG with time measured.

The results showed that at all periods the palmitate-1-¹⁴C radioactivity of plasma TG of retinol fed rats was consistently higher than the control (Fig. 5). The specific activities (counts/min/mg) of plasma TG of control and retinol fed rats (Fig. 6) show that the turnover rate of plasma TG in retinol fed rats was slower than that of the controls. These results point out an impairment in the uptake of plasma TG in retinol fed rats by extrahepatic tissues. Intravenous administration of Triton into animals causes marked hyperlipemia and hypertriglyceridemia.^{23~24)} This effect results from a block in the exit of lipids from plasma compartment due to alterations in the physicochemical properties of plasma lipoproteins.²⁵⁾ From the results shown in Fig. 1, it is seen that in control animals 60 min after the administration of Triton, plasma TG increased by about two fold whereas no such increase was noted in retinol fed rats. Possible explanation for this could be that retinol fed rats were already hypertriglyceridemic and thus only a slight increase in their plasma TG after the administration of Triton could be observed. The hyperlipemia and hypertriglyceridemia observed in retinol fed rats could also result from their decreased utilization by extrahepatic tissues.

In experimental conditions where an increased accumulation of TG in liver has been reported, an altered metabolism of liver phospholipids has also been observed.⁷⁾ In retinol fed rats liver contents of phosphatidyl choline were significantly higher and that of phosphatidyl ethanolamine unchanged as compared to control (Table I). Results of incorporation of palmitic acid-1-¹⁴C into phosphatidyl choline (percent injected dose) show that extent of the label in PC of retinol fed rats were significantly higher than the controls (Fig. 7). However the specific activity of liver PC (counts/min/mg) of retinol fed rats was considerably lower than the controls (Fig. 8) indicating that its synthesis from palmitate-1-¹⁴C was reduced in retinol fed rats and the increased radioactivity observed in PC (percent injected dose) in retinol fed rats (Fig. 7) results from their

impaired secretion into plasma. Both the total palmitate-1-¹⁴C radioactivity (percent injected dose, Fig. 9) and the specific activity (counts/min/mg) of liver PE (Fig. 10) in retinol fed rats were higher than the control. These results suggest that the total activity of PE may result from its impaired secretion as well as slower turnover rate in liver of retinol fed rats as compared to the controls. Both PC and PE are the major phospholipids of liver constituting about 70~80 percent of liver total phospholipids. In plasma, PC forms about 60 percent of plasma phospholipids which are derived from liver, but PE forms only a small fraction of plasma phospholipids. Therefore, the measurement of the secretion of plasma PC in Tritonized rats could also provide an indirect information on hepatic TG secretion.⁷⁾ The results showed that in control rats 60 min after the administration of Triton there was a 38 percent increase in plasma PC, whereas no such increase was noted in retinol fed rats (Fig. 1). Like plasma TG, the values of plasma PC were about 2 fold higher in retinol fed and tritonized rats as compared to control (Fig. 1). Therefore, due to already expanded pool of plasma PC in retinol fed rats any increase in plasma PC due to Triton administration may not be evident. These results suggest an impaired secretion of liver TG and PC into plasma of retinol fed rats. The increased levels of TG and PC may have resulted from their impaired uptake by extrahepatic tissues. The total activity (percent injected dose) of palmitate-1-¹⁴C appearing in plasma PC of retinol fed rats was higher than the control (Fig. 11), but the specific activity was lower than the control (Fig. 12). The former results suggest a slower loss of the PC label in plasma of retinol fed rats (Fig. 11), perhaps by its impaired uptake by extrahepatic tissues, resulting in the accumulation of newly secreted labelled PC in plasma of retinol fed rats. The lowered specific activity of plasma PC in retinol fed rats results from its expanded pool size. It may be noted that there are differences between increase in plasma TG and PC content in control tritonized rats. There was about

two fold increase in TG, whereas in PC only 38 percent increase was noted (Fig. 1). The reasons for this are a considerably slower turnover of plasma PC and the fact that among the plasma lipoproteins the very low density lipoproteins fraction has the fastest turnover but the lowest content of phospholipids.

Results of this study show that the elevated levels of triglycerides in liver of retinol fed rats as compared to control result from their increased synthesis and impaired secretion into plasma. Hepatic triglycerides are synthesized from fatty acids and α -glycerophosphate.²⁷⁾ The rate of synthesis is directly proportional to the concentration of both the substrates.²⁸⁻³⁰⁾ The significantly elevated levels of plasma-non esterified fatty acids in retinol fed rats as compared to control (Table I) show that liver utilizes fatty acids for TG synthesis which are mobilized from adipose tissues. Hence in conditions of extensive lipolysis it is reasonable to assume that the flux of free fatty acid to liver is enhanced.³¹⁾ Evidence to this assumption is provided by the specific activities (counts/min/mg) data of liver TG in control and retinol fed rats (Fig. 4), which were not different, inspite of the fact that the palmitate-1-¹⁴C radioactivity of plasma free fatty acids (precursor pool of liver fatty acids) in retinol fed rats was considerably diluted due to its expanded pool size as compared to the control (Table I). Under these conditions, to achieve the synthetic rate of liver TG in retinol fed rats similar to that of control, the flux of plasma free fatty acids to liver in retinol fed rats has to be more. The amounts of α -glycerophosphate needed for TG synthesis may be provided by glycolytic and/or gluconeogenic pathway or by the glycerokinase reactions.³²⁻³³⁾ Corticosteroids have been implicated to be involved in the liver triglyceride metabolism. Corticosteroid administration caused increased synthesis of triglycerides in liver and produced hypertriglyceridemia.^{34, 35)} Studying protein synthesis in liver³⁶⁾ and cholesterologenesis in adrenals of rats given vitamin A and vitamin C,^{37, 38)} we have suggested that the effect of vitamin A on liver lipid metabolism are mediated through

the adrenals. The results of the present investigation further suggest that the effects of vitamin A on liver triglycerides are mediated through the adrenals.

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