Sources of Triacylglycerol Accumulation in Livers of Rats Fed a Cholesterol-Supplemented Diet

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ABSTRACT: The source of free fatty acids (FFA) and the pathways contributing to the accumulation of neutral fats in livers of rats fed a cholesterol-enriched diet were investigated in this report. Supplementation with 1% cholesterol in the diet for four weeks resulted in hepatomegaly in the rats. The contents of cholesterol and triacylglycerols (TG) per gram liver measured in rats fasted overnight increased by 48 mg (~tenfold) and 66 mg (~fourfold), respectively. The activities of glycerophosphate acyltransferase and diacylglycerol acyltransferase, the two key enzymes for TG synthesis in liver microsomes, were found to increase by 23 and 19%, respectively, in the cholesterol-fed rats. The secretion of plasma TG present predominantly in very low density lipoprotein was found to decrease by ~30%. The incorporation of tritium from tritiated water in liver FFA increased by twofold in rats fed the cholesterol-supplemented diet, whereas the activity of CPT I in liver mitochondria decreased by 23%. The uptake of plasma FFA *in vivo* in livers of fasted rats maintained on the cholesterol-supplemented diet decreased by 60%. Our data thus indicate that the excess TG accumulated in livers of rats fed the cholesterol-enriched diet resulted from increased synthesis and decreased secretion of TG. To meet the demand of fatty acids for this purpose, *de novo* lipogenesis increased, whereas β-oxidation decreased. Although difference in the uptake of extrahepatic FFA may be discounted, a difference in the uptake of chylomicron remnants between the control and cholesterol-fed rats may not be ruled out.
Lipids 30, 527–531 (1995).

We have previously found that rats fed diets enriched in cholesterol often developed moderate to severe fatty livers (1,2). Cholesterol and triacylglycerols (TG) were found to accumulate in the liver (1,2). It has been suggested that the etiology of the fatty liver is related to essential fatty acid (EFA) deficiency (3), as linoleic acid was shown to be a preferred substrate for the esterification of cholesterol (4). EFA deficiency may impair the synthesis and function of phospholipids (PL) in the liver. Consequently, the ability to assemble and secrete very low density lipoprotein particles (VLDL) may be impaired. Recent reports by Fungwe *et al.* (5–7), however, showed that cholesterol-induced fatty liver was not necessarily related to EFA deficiency. Supplementing the cholesterol diets with a large quantity of corn oil (5–20%), a vegetable oil rich in linoleic acid, failed to prevent the accumulation of excess TG in the liver (6). Furthermore, the secretion of VLDL from the perfused liver was shown not to decrease, but rather to increase, in rats fed cholesterol-supplemented diets (7).

TG accumulation in the liver may be caused by increased synthesis of TG in the liver and/or decreased secretion of TG from the liver (5). The availability of free fatty acids (FFA) in the hepatocytes may be increased through an increase in *de novo* synthesis (7), an increase in uptake from extra-hepatic sources (6), and/or a decrease in β-oxidation (6). In this report, the roles of these metabolic pathways in contributing to cholesterol-induced fatty liver were characterized.

MATERIALS AND METHODS

Chemicals. L-[2-^{3}H]Glycerol 3-phosphate, disodium (10.6 Ci/mmol), [1-^{14}C]palmitoyl CoA (60 mCi/mmol), and tritiated water (1 Ci/mL) were purchased from Du Pont/New England Nuclear (Boston, MA). L-[methyl-^{3}H]Carnitine hydrochloride (74 Ci/mmol) was purchased from Amersham Corp (Arlington Heights, IL). Enzyme kits for the determination of cholesterol, TG, and FFA were purchased from Boehringer Mannheim (Mannheim, Germany). The kit for the determination of PL was purchased from bioMerieux Co. (Marcy-l'Etoile, France). Triton WR-1339 was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade.

Animals and diets. Male Wistar rats (190–210 g) were housed in a room with a 12 h light–dark cycle (light period: 7 a.m. to 7 p.m.). The animals were divided into two groups—one was fed a cholesterol-supplemented diet (1%) and the other the control diet for four weeks. The compositions of the control and cholesterol-supplemented diets are shown in Table 1. Lard and soybean oil were proportioned in the diets to yield a polyunsaturated/saturated fatty acid ratio of 1. De-

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Abbreviations: DGAT, diacylglycerol acyltransferase; EFA, essential fatty acids; FFA, free fatty acids; GAT, glycerophosphate acyltransferase; PL, phospholipids; TG, triacylglycerols; VLDL, very low density lipoproteins.

TABLE 1
Composition of the Control and Cholesterol-Supplemented Diets

Ingredients	Control diet (%)	Cholesterol-supplemented diet (%)
Casein	20.0	20.0
Methyl cellulose	3.0	3.0
Vitamin mixture[a]	1.0	1.0
Salt mixture[a]	3.5	3.5
Choline chloride	0.095	0.095
Cholesterol	0	1.0
Lard	7.0	7.0
Soybean oil	3.0	3.0
DL-methionine	0.3	0.3
Sodium cholate	0.3	0.3
Corn starch	(making up to 100%)	

[a]AIN 76 vitamin and mineral mixtures.

pending on the protocol, the rats were either fed or fasted overnight at the time of the experiment. For rats fasted overnight, the food was removed at 5 p.m. and the experiment started at ~9 a.m. on the next day. The animals were allowed free access to drinking water at any time prior to the experiment.

Lipid analyses. Rats fasted overnight were sacrificed by decapitation. Blood was collected in tubes containing EDTA (1 mg/mL). Livers were excised and placed in ice-cold saline. The weight of the liver was determined after blotting dry. The livers were minced. Total lipids in the liver were isolated by the method of Folch *et al.* (8). The concentrations of cholesterol, PL, and TG were determined by the methods of Abell *et al.* (9), Stewart (10), and Soloni (11), respectively. Plasma was separated from blood cells by centrifugation. Plasma FFA were measured enzymatically (12).

Enzyme assays. Liver mitochondria were isolated from fed rats according to Harper and Saggerson (13). The microsomal fraction of liver homogenates was isolated according to Erickson *et al.* (14). The activity of mitochondrial CPT I was assayed by measuring the formation of [^{3}H] palmitoylcarnitine from [^{3}H] carnitine and palmitoyl CoA according to Saggerson *et al.* (15). The activity of microsomal diacylglycerol acyltransferase (DGAT) was measured by following the formation of [^{14}C] TG from [^{14}C] palmitoyl CoA and diacylglycerol according to Coleman and Bell (16). The microsomal activity of glycerol acyltransferase (GAT) was assayed by following the formation of [^{3}H]1-acylglycerol 3-phosphate from [^{3}H]glycerol 3-phosphate and palmitoyl CoA as described by Aarsaether *et al.* (17). Protein was determined by the method of Lowry *et al.* (18).

De novo synthesis of FFA. Fed rats were injected with 30 mCi [^{3}H]H_2O intraperitoneally one hour before sacrifice. *De novo* synthesis of FFA was measured according to Venkatesan *et al.* (19). Briefly, lipids were extracted from the liver and separated on thin-layer chromatography plates (Silica gel G) using a solvent system consisting of *n*-hexane, diethyl ether, and formic acid (80:20:2). Lipid fractions were visualized in an iodine chamber. FFA fractions were identified and scraped off the thin-layer chromatography plates. Radioactivity in FFA was determined in a Beckman (Palo Alto, CA) LS-250 β-counter.

TG secretion. A 20% solution of Triton WR-1339 was intravenously injected (50 mg/100 g body weight) to inhibit lipolysis of TG in the rats in the fasted state (20). The rats were sacrificed two hours after the injection as described by Swift *et al.* (21). Blood plasma was collected and VLDLs ($d < 1.006$ g/mL) were isolated by ultracentrifugation. Cholesterol, PL, and TG in VLDL, as well as in the unfractionated plasma, were measured by methods described previously.

Hepatic uptake of FFA. The uptake of plasma FFA in the liver *in vivo* was measured by an arteriovenous technique as described by Huang *et al.* (22). The animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg). Polyethylene cannulas (PE 10) were implanted in the aortas, portal veins, and hepatic vein (22). While the animals were being anesthetized, blood samples were taken sequentially from the hepatic veins, portal veins, and aortas through the implanted cannula into 1 c.c. tuberculin syringes previously rinsed with 5% EDTA. Hepatic uptake of FFA was determined by subtracting the concentration of FFA in the hepatic vein from that calculated for the hepatic influx (22). Hepatic influx concentration of FFA ($[FFA]_{inf}$) was calculated from the sum of the concentrations of FFA in the aorta ($[FFA]_a$), substituting for the hepatic artery, and the portal vein ($[FFA]_p$) multiplied by their respective fractional contributions of blood supply to the liver (i.e., $[FFA]_{inf} = 0.3 \times [FFA]_a + 0.7 \times [FFA]_p$). The fractional contributions of blood supply to the liver from the portal vein and the hepatic artery were previously determined to be 0.7 and 0.3, respectively (23). The purity of blood taken from the hepatic vein, free from vena caval contamination, was determined by the $p0_2$ method according to Huang (24).

Statistical analyses. Data were expressed as means ± SD. Statistical significance between data from rats fed the control and cholesterol-supplemented diets was determined by Student's *t*-test. A P value less than 0.05 was considered statistically significant.

RESULTS

Body and liver weight. Feed efficiency of the control and the cholesterol-supplemented diets was about the same. The gain in body weight was not significantly different between the two groups for a period of up to four weeks. Liver weight was, however, significantly increased in the group fed the high-cholesterol diet (12.4 ± 1.0 g vs. 8.4 ± 1.1 g in the control). Consequently, liver-to-body weight ratio increased in this group of rats.

Liver lipids. The content of cholesterol in the fasted state increased from ~5 mg/g liver in the control to ~53 mg/g liver in the cholesterol-fed rats (Table 2), and the content of TG increased from ~25 mg/g liver to ~90 mg/g liver (Table 2). The increases of cholesterol and TG in the liver were approximately ten- and fourfold, respectively, in the cholesterol-fed rats as compared to the controls. PL, on the other hand, de-

TABLE 2
The Contents of Cholesterol, Triacylglycerols, and Phospholipids in Livers of Rats That Had Been Fed Control or Cholesterol-Supplemented Diets for Four Weeks[a]

Diet	Cholesterol (mg/g liver)	Triacylglycerols (mg/g liver)	Phospholipids (mg/g liver)
Control	4.8 ± 0.5	24.5 ± 6.2	31.7 ± 2.3
Cholesterol	53.1 ± 7.8[b]	90.3 ± 16.9[b]	26.9 ± 2.3[b]

[a]Data are means ± SD of six experiments for both groups, which were fasted overnight prior to sacrifice.
[b]$P < 0.05$, vs. control.

creased (13%) on a per gram liver basis in the cholesterol-fed rats, as compared to the controls (Table 2). On the whole liver basis, cholesterol feeding increased the content of cholesterol in the liver by 14.6-fold, TGs by 5.1-fold, and PLs by 1.3-fold.

TG synthesis. The activities of the two key enzymes involved in TG synthesis, GAT and DGAT, were measured in liver microsomes isolated from rats fed the control and the cholesterol-supplemented diets in the fed state. The activity of GAT, which catalyzes the first esterification step of TG synthesis, increased 23% in the cholesterol-fed rats (Table 3). The activity of DGAT, which catalyzes the conversion of diacylglycerols to TG, increased by 19% in the cholesterol-fed rats, as compared to the controls (Table 3).

β-Oxidation of FFA. The activity of CPT I in isolated mitochondria, the rate-limiting enzyme for β-oxidation, was found to decrease significantly by 23% in the fed state in rats fed the cholesterol-supplemented diet, as compared to the controls (Table 3).

De novo *synthesis of FFA.* The incorporation of tritiated water injected intraperitoneally in fatty acids in the liver was 6,544 ± 1,480 dpm/h/g in the control and 13,240 ± 2,663 dpm/h/g in the cholesterol-fed rats. The rate of *de novo* synthesis of FFA thus increased by twofold in the cholesterol-fed rats as compared to the controls.

Hepatic secretion of lipids. Total and VLDL lipid secretion were measured in the fasted state. Triton WR-1339 was injected intravenously to block the activity of lipoprotein lipase. The contents of lipids in the unfractionated plasma and in VLDL before and after two hours of injection are shown, respectively, in Tables 4 and 5. No significant difference in

TABLE 3
Activities of Microsomal Glycerophosphate Acyltransferase (GAT), Diacylglycerol Acyltransferase (DGAT), and CPT-I in Livers of Rats Fed Control or Cholesterol-Supplemented Diet for Four Weeks[a]

Diet	GAT[b]	DGAT[c]	CPT-I[d]
Control	1.13 ± 0.16	3.36 ± 0.36	9.67 ± 1.06
Cholesterol	1.39 ± 0.08[e]	4.01±0.58[e]	7.46 ± 0.84[e]

[a]Data are means ± SD. Numbers of experiments in each groups are 8.
[b]Expressed as nmol of 1-acylglycerol 3-phosphate formed/min/mg microsomal protein.
[c]Expressed as nmol of triacylglycerol formed/min /mg microsomal protein.
[d]Expressed as nmol of palmitoyl–CoA formed/min/mg mitochondrial protein.
[e]$P < 0.05$, vs. control.

TABLE 4
Total Triacylglycerols, Cholesterol, and Phospholipids in Plasma Before and After Injection of Triton WR-1339 in Rats That Had Been Fed Control or Cholesterol-Supplemented Diet for Four Weeks[a]

Diet	Control	Cholesterol-fed
Triacylglycerols		
Before	104.7 ± 39.0	80.5 ±17.4
After	785.1 ± 86.9	574.8 ± 88.8[b]
Cholesterol		
Before	82.5 ± 10.5	85.5 ± 10.5
After	156.6 ± 17.2	345.2 ± 24.2[b]
Phospholipids		
Before	126.5 ± 13.0	105.6 ± 6.2[b]
After	91.7 ± 12.9	182.5 ± 23.4[b]

[a]Data are means ± SD. Units are mg/dL. Rats from each dietary group were divided into two groups for the experiments—after fasting overnight, one was sacrificed before, and the other two hours after intravenous injection of Triton WR-1339 (50 mg/100 g body weight). Numbers of experiments for both the control and cholesterol-fed rats were six before the injection, and eight after the injection.
[b]$P < 0.05$, vs. control.

TG concentration was found between VLDL and the unfractionated plasma before the injection of Triton in the two groups. After the injection, plasma TG, found to be associated predominantly with VLDL, increased approximately sevenfold in both groups of rats. The rate of increase in plasma TG concentration in the cholesterol-fed rats was, however, ~40% less than that in the controls. VLDL cholesterol was 13% of the total in the plasma in the control and 29% in the cholesterol-fed rats before the injection of Triton. The proportion of plasma cholesterol in VLDL increased to 60% in the cholesterol-fed and 72% in the control rats after the injection of Triton. The increases in the concentration of cholesterol were 1.9–4.0-fold in the plasma and 8.2–11-fold in VLDL after the injection of Triton. VLDL–PL as a percentage of total plasma PL was ~15% in the two groups before

TABLE 5
VLDL-Triacylglycerols, Cholesterol, and Phospholipids Before and After Injection of Triton WR-1339 in Rats That Had Been Fed Control or Cholesterol-Supplemented Diet for Four Weeks[a]

Diet	Control	Cholesterol-fed
Triacylglycerols		
Before	78.0 ± 32.1	58.0 ±14.0
After	774.5 ± 86.9	550.0 ± 85.3[b]
Cholesterol		
Before	10.9 ± 4.1	25.2 ± 9.4[b]
After	112.2 ±18.5	207.5 ± 3.3[b]
Phospholipids		
Before	16.4 ± 6.4	18.2 ± 5.5
After	112.4 ± 9.5	139.6 ± 11.1[b]

[a]Data are means ± SD. Units are mg/dL. Rats from each dietary group were divided into two groups for the experiments—after fasting overnight, one was sacrificed before, and the other two hours after intravenous injection of Triton WR-1339 (50 mg/100 g body weight). Numbers of experiments for both the control and cholesterol-fed rats were six before the injection, and eight after the injection.
[b]$P < 0.05$, vs. control.

the injection. After the injection, the concentration was not changed in the control, but was increased 73% in the cholesterol-fed rats. VLDL–PL as a percentage of the total in the plasma was increased to 75% in the cholesterol-fed rats and 100% in the control rats after the injection of Triton.

Hepatic gradients of FFA. The uptake of extra-hepatic FFA in the liver was measured by arteriovenous difference technique in intact rats (22). In the fasted state, the concentration of FFA in the aorta was 0.74 ± 0.17 mM (n = 5) in rats fed the cholesterol-supplemented diet and 0.96 ± 0.19 mM (n = 4) in rats fed the control diet. The difference between the two was not statistically significant. The arteriovenous gradient of FFA across the liver in the control rats was, however, 100% greater than that found in the cholesterol-fed rats (0.78 ± 0.37 vs. 0.39 ± 0.18 mM). This indicates that the uptake of plasma FFA in the liver was 50% less in rats fed the cholesterol-supplemented diet than the controls in the fasted state, assuming that hepatic blood flow was not significantly different between the two groups.

DISCUSSION

In this study, we showed that supplementation of 1% cholesterol in the diet for four weeks stimulated the synthesis and accumulation of TG in the liver. Because the polyunsaturated/saturated fatty acid ratio of the dietary fat was set to be 1, following the dietary guideline of the National Science Council (Washington, D.C.), the supply of the polyunsaturated fatty acids to the rats was deemed sufficient. The incidence of the fatty liver was thus not a result of a deficiency in EFA (3,4) or a result of impaired PL synthesis (3,4), as was suggested by the diminished PL content in rats fed the cholesterol-supplemented diet on the basis of per gram tissue weight (Table 2). On the basis of the whole liver, PL content in the cholesterol-fed rats was found to increase by 30%. Furthermore, the secretion of PL in VLDL was found to increase in rats fed the cholesterol-supplemented diet.

The amounts of TG and cholesterol accumulated in the liver in the current study were three- to fivefold greater than those reported by Fungwe *et al.* (6). These discrepancies may be attributed to differences in the dietary regimen in the two studies and the duration during which the animals were maintained on the diets. Fat content in our diet was 10% rather than 5% as in the study of Fungwe *et al.* (6). Cholesterol contents in the diets in our study and that of Fungwe *et al.* (6) were 1 and 0.5%, respectively. The animals in our study were fed the diet for four weeks, while those of Fungwe *et al.* (6) were fed for one week. According to Fungwe *et al.* (6), changing dietary fat content alone from 5 to 15% can result in a threefold increase in liver TG and a twofold increase in liver cholesteryl esters.

While Fungwe *et al.* (5) showed that the secretion of VLDL-TG increased in the isolated perfused liver, we found that *in vivo* the secretion of TG by the liver determined in the plasma or VLDL decreased in rats fed the cholesterol-supplemented diet, as compared to the controls. Plasma TG was found predominantly in VLDL both before and after the injection of the inhibitor for lipoprotein lipase, Triton WR 1339, in either the control or cholesterol-fed rats (Tables 4 and 5). Very little TG was found to be associated with lipoproteins other than VLDL.

In contrast, the concentration of cholesterol increased 3.5-fold in the plasma and 1.8-fold in VLDL in the cholesterol-fed rats, as compared to the controls (Tables 4 and 5). The majority of the cholesterol in the plasma was found to be present in lipoproteins other than VLDL before the injection of Triton WR 1339. After the injection, the percentage of cholesterol in VLDL increased. A significant proportion of plasma cholesterol remained in lipoprotein fractions other than VLDL. The rate of accumulation of cholesterol in non-VLDL lipoproteins was greater in the cholesterol-fed rats than in the controls, suggesting that the turnover of cholesterol and cholesteryl esters in non-VLDL lipoproteins was increased in the cholesterol-fed rats.

Like cholesterol, the secretion of PL also increased in the cholesterol-fed rats (Tables 4 and 5). Blockade of the activity of lipoprotein lipase resulted in no additional PL secretion in the control, but about 73% more PL in the cholesterol-fed rats. The percentage of plasma PL in VLDL increased in both groups of rats following the injection of Triton. Before the injection of Triton WR 1339, only a small fraction of plasma PL was found to be associated with VLDL. After the injection, almost all of the PL in the control and 75% in the cholesterol-fed rats was found in VLDL. Thus, it seems that inhibition of lipoprotein lipase could result in the redistribution of PL in the lipoproteins.

No attempt was made to differentiate the unesterified and esterified forms of cholesterol in this study. Given the magnitude of the increase in total cholesterol in VLDL (85%), both forms of cholesterol can be expected to increase. Fungwe *et al.* (5) showed that the increase in plasma cholesterol can be found mainly in the esterified rather than the unesterified fraction of cholesterol. Because the increases in the amounts of surface lipids (PL and cholesterol) in the cholesterol-fed rats were small (~10%), VLDL from this group of rats and that from the control rats were not expected to be different in size. Both TG and cholesteryl esters are located in the core of VLDL particles. Constrained by particle size, excess secretion of cholesteryl esters may result in less space for TG to be incorporated in VLDL (25). This has been demonstrated in *in vitro* experiment. When isolated hepatocytes were incubated in a medium enriched in cholesterol, the secretion of cholesteryl esters increased and TG decreased in VLDL (26).

The other question to be addressed in this report is the source of FFA for TG synthesis in the liver. We found that *de novo* synthesis of FFA increased, and the degradation of FFA in the liver decreased in rats fed the cholesterol-supplemented diet, in agreement with the findings of Fungwe *et al.* (5–7). In contrast to the experiment of Fungwe *et al.* (6), hepatic uptake of FFA was measured *in vivo* instead of *in vitro* in the isolated perfused liver (6). Whereas the uptake of plasma FFA in the isolated perfused liver was not affected by

dietary cholesterol (6), the uptake *in vivo* as indicated by the arteriovenous gradient of FFA across the liver decreased by 60%. Both studies nevertheless ruled out plasma FFA as a source for excess TG synthesis in the liver. It appears that the *de novo* synthesized FFA may be sufficient to account for the TG accumulated in the liver in the cholesterol-fed rats. As the supply of cholesterol is abundant, it is not necessary to maintain 3-hydroxy-3-methylglutaryl–CoA reductase in an active state. More acetyl CoA may be available for lipogenesis (7). To prevent futile cycling between lipogenesis and FFA oxidation, the activity of mitochondrial CPT I diminished (6).

Another source of extrahepatic FFA for TG accumulation in cholesterol-fed rats is cholesteryl esters contained in the hydrophobic cores of apolipoprotein C-depleted VLDL and chylomicron remnants (27). Cholesteryl esters in these lipoprotein particles can be transferred preferentially to the liver through interactions at the cell surface between these particles, hepatic lipase, and low-density lipoprotein receptor before the irreversible uptake of the lipoprotein particles themselves (27,28). The half-life of cholesteryl esters in the liver is short (~15 min) (27). Fatty acids released through the hydrolysis of cholesteryl esters in the liver were found to be incorporated in glycerolipids (27). The exact role for this pathway in TG accumulation in the cholesterol-fed rats was not determined in this report.

The increases in the activities of GAT and DGAT in livers of the cholesterol-fed rats may be secondary to the increase in fatty acid synthesis. The increases in DGAT activity and TG synthesis in primary culture of hepatocytes were found to be associated with an increase in the availability of FFA in the medium (29). Our data from the arteriovenous studies, however, indicate that newly synthesized FFA is more effective than plasma FFA in serving as the substrate for TG synthesis in the liver. The effectiveness of endogenous FFA in promoting TG synthesis has also been shown by Fungwe *et al.* (6). These investigators found that although the incorporation of [^{14}C] oleate in TG increased in the isolated perfused liver, the uptake of [1-^{14}C] oleate was not significantly altered in the cholesterol-fed rats.

In conclusion, our results support the roles of increased lipogenesis, decreased oxidation of fatty acids and decreased secretion of VLDL as causes for the accumulation of TG in the liver in the cholesterol-fed rats. Of the two extra-hepatic sources of fatty acids, albumin-bound FFA may be discounted for the difference in TG accumulation in the livers between the control and cholesterol-fed rats. A difference in the uptake of esterified fatty acids in chylomicron remnants, however, remains a possibility.

ACKNOWLEDGMENTS

We would like to thank C.C. Chao for technical assistance. This work was supported by grants (NSC-80-0412-B002-42, NSC-81-0412-B002-112, and NSC-82-0412-B182-26) from the National Science Council of the ROC government in Taiwan.

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[Received September 14, 1994, and in revised form March 13, 1995; Revision accepted April 28, 1995]