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EFFECT OF CLOFIBRATE ON THE HANDLING OF DIETARY AND LIVER FAT*

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Abstract—*p*-Chlorophenoxyisobutyrate (CPIB) had a marked effect on the handling of erogenous fat by rats. After an oral fat load, the serum triglyceride response was plotted, and the areas under such curves were compared with the amounts of α-glycerophosphate dehydrogenase (GPD) and malic enzyme present in the liver. CPIB and L-thyroxine (T_4) increased these enzyme activities; diphenylthiohydantoin and imidazole were also used to inhibit the induction of GPD and enhance the malic enzyme response respectively. Changes in these enzyme activities did not appear to be involved in the CPIB effect on the disposition of oral fat. CPIB completely prevented or corrected the fatty liver produced by feeding orotic acid to intact or thyroidectomized rats, and gave a strong β-lipoprotein band in serum gel electrophresis; T_4 had only a small effect. The effect of CPIB on fat metabolism was therefore not mediated through its enhancement of T_4 effects in the liver.

A VARIETY of effects have been demonstrated for hypolipidemic drugs such as clofibrate (CPIB).† In the intact rat, CPIB produces hepatomegaly with an increased number of microbodies;[1,2] it activates lipoprotein lipase,[3] decreases liver triglyceride (TG)[4] and plasma free fatty acids,[5,6] and displaces substances such as thyroxine (T_4)[7–9] and warfarin[10,11] from plasma proteins. It produces hyperthyroid effects in the liver, which increase the α-glycerophosphate dehydrogenase (GPD) and malic enzyme.[12,13] *In vitro*, CPIB inhibits fatty acid[14] and cholesterol synthesis[15,16] and increases the esterification of cholesterol by rat liver microsomes.[17] Much of the literature has been reviewed in a recent symposium.[18–22] Which of these effects is responsible for the hypolipidemic action of the drug has yet to be established.

The studies reported here-in were planned to evaluate any possible role of liver GPD and malic enzyme in the CPIB effect on fat metabolism. CPIB had a marked effect on the fat tolerance curve produced by a standardized fat load test, and this was used for comparison with the effect of an equivalent amount of T_4. Both enzymes increased simultaneously after either T_4 or CPIB administration, but the responses of the two enzymes were separated by 5,5′-diphenyl-2-thiohydantoin (DPTH) and imidazole. DPTH blocked the GPD response much more than the malic enzyme response to T_4.[23] Imidazole increased the malic enzyme *per se* and this was additive with the T_4 effect. By the use of these substances along with T_4 and CPIB, widely varying enzyme activities were produced in the liver. The latter were then compared with the effects of CPIB on the fat tolerance curve.

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† Clofibrate (*p*-chlorophenoxyisobutyrate) was obtained from Ayerst Laboratories.

Orotic acid blocks the synthesis of pre-β-lipoproteins[24-26] and produces a fatty liver by blocking the exit of fat from the liver. Orotic acid was used in these studies in an effort to determine the role of CPIB on fat metabolism in the liver. The results showed that CPIB prevented or corrected the orotic acid fatty liver by by-passing the pre-β-block. Hess *et al.*[27] previously reported that the Ciba drug, Su 13437* prevented an orotic acid fatty liver. The characteristic feature of feeding CPIB under all the experimental conditions tested was the appearance of a strong β-lipoprotein band in the serum gel electrophoresis. Presumably, the liver lipids which accumulated in the presence of orotic acid were removed from the liver as β-lipoproteins when the rats were fed CPIB.

METHODS

Male rats (200 g; R. Miller, Cazenovia, N.Y.) were fed a synthetic diet containing (as per cent of the diet): casein, 28; corn oil, 10; glucose, 58; salts, 4; plus a complete vitamin mixture. The various drugs were added to this basal diet in the following concentrations (as per cent of the diet): orotic acid, 1·0; CPIB, 0·3, adenine, 0·25, imidazole, 0·2, Ciba compound Su 13437, 0·1; DPTH, 0·06. T_4 was injected subcutaneously at 6 μg/100 g body weight/day. After 2 weeks on the various diets, the rats were fasted overnight, given 0·25 ml corn oil/100 g body weight by stomach tube, and groups of rats were sacrificed by decapitation at 0, 2, 4, 6 and 8 hr thereafter. Serum and liver samples were analyzed for TG[28] and cholesterol,[29] and the area under each serum TG tolerance curve was measured by a planimeter. Liver GPD and malic enzyme were determined as previously described.[30,31] Serum lipoprotein patterns were determined by gel electrophoresis (Quick-Disc electrophoresis of lipoproteins, Canalco Diagnostic Products, Rockville, Md.).

RESULTS

The liver GPD and malic enzyme activities which were obtained under the different experimental conditions are given in Table 1. The amount of T_4 administered increased these activities on the basal diet to the same extent as the CPIB. DPTH inhibited the effect of T_4 and CPIB on liver GPD much more than it inhibited the malic enzyme response. Imidazole *per se* increased the malic enzyme activity, and this was additive with the T_4, but not the CPIB effect; it had relatively little effect on liver GPD. Orotic acid did not inhibit the GPD response to T_4 or CPIB, but it inhibited the endogenous malic enzyme as well as the malic enzyme response to T_4.

Figure 1 shows typical fat load tests obtained in these experiments. T_4 did not lower the fasting serum TG, but it decreased the tolerance curve somewhat. CPIB not only decreased the fasting serum TG, but it had a marked effect on the handling of an exogenous fat load. Orotic acid also hastened the removal of serum TG somewhat, and this could be due to a trapping of some of the dietary fat in the liver when the latter was unable to secrete pre-β-lipoproteins.

When the areas under such tolerance curves were plotted against the corresponding GPD or malic enzyme activities, the results shown in Fig. 2 were obtained. DPTH held the liver GPD to normal or lower values, but had an unexpected effect on the handling of exogenous fat; later studies showed that it also prevented the fatty liver induced by

* Su 13437 [2-methyl-2-(*p*-1,2,3,4-tetrahydro-1-naphthylphenoxy) propionic acid] was obtained from Ciba Pharmaceutical Company.

TABLE 1. LIVER ENZYME ACTIVITIES UNDER VARIOUS EXPERIMENTAL CONDITIONS*

	No treatment		+ T_4 (6 μg/100 g of body wt. /day)		+ CPIB (0·3% of the diet)	
Diet	GPD†	Malic‡	GPD	Malic	GPD	Malic
Basal	23 ± 0·7§	1·4 ± 0·05§	75 ± 2·4§	4·8 ± 0·20§	71 ± 2·3‖	5·0 ± 0·19‖
DPTH (0·06)	9 ± 0·5‖	2·0 ± 0·08‖	22 ± 0·1‖	3·8 ± 0·5‖	17 ± 1·0	4·3 ± 0·32
Imidazole (0·2)	20 ± 1·4‖	3·7 ± 0·18‖	61 ± 2·6‖	7·9 ± 0·43‖	57 ± 3·2	5·7 ± 0·38
Orotic acid	14 ± 0·6	0·5 ± 0·03	84 ± 4·0	3·4 ± 0·18	74 ± 4·1	4·6 ± 0·20

* Male rats (200 g) were fed the various drugs (as per cent of the diet) and/or injected daily with T_4 subcutaneously for 2 weeks. Mean ± S.E. for groups of approximately 24 rats each, except as noted below.

† GPD = microliters of O_2/10 min/150 mg of fresh liver.

‡ Malic enzyme = micromoles of TPNH/minute/gram of fresh liver.

§ There were 72 rats in this group.

‖ There were 48 rats in this group.

orotic acid. The results show that an increased liver GPD is not essential for the alteration of a fat load test by DPTH, but provide no evidence on the role of GPD in the CPIB effect. The results also show that the increased malic enzyme activity produced by imidazole does not increase the handling of an exogenous fat load.

Orotic acid. Figure 3 shows the accumulation of liver TG and cholesterol when rats were fed 1 per cent orotic acid in diets containing 10 per cent corn oil (21 per cent

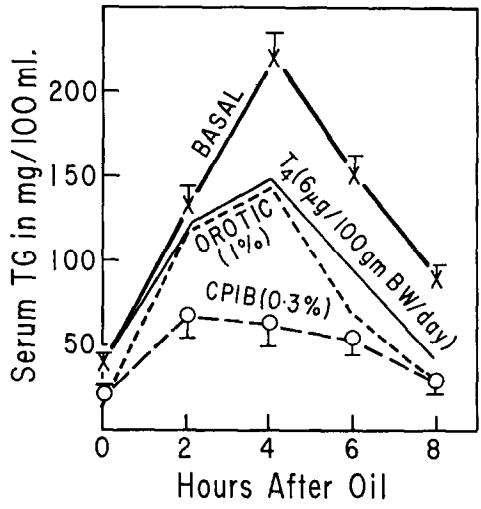

FIG. 1. Typical fat tolerance curves. Male rats (200 g) were fed the various diets or injected with T_4 for 2 weeks, fasted overnight, given 0·25 ml corn oil/100 g of body weight by stomach tube at 0 time, and serum triglycerides were determined at 0, 2, 4, 6 and 8 hr later. Mean ± S.E. has been shown for groups of 25 rats on the basal diet and 10 rats per point on CPIB.

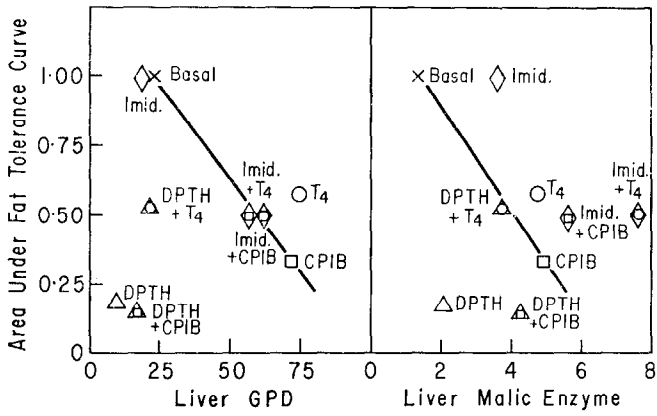

FIG. 2. Fat tolerance curves in relation to liver GPD or malic enzyme activities. The area under the basal fat tolerance curve as illustrated in Fig. 1 was assigned a value of 1·0, and other areas were calculated as a fraction of this. The corresponding liver enzyme activities are given in Table 1. The lines represent the theoretical relationships which would obtain if the enzyme changes were responsible for the CPIB effect.

of the calories) or no fat (except for 5 per cent of the calories as ethyl linoleate). Both TG and cholesterol began to accumulate after 4 days and reached a plateau after about 4 weeks at 130–180 mg/g of TG and 17–19 mg/g of cholesterol on the 10 per cent fat diet. During the plateau phase, the serum was still deficient in pre-β-lipoproteins and contained little TG or cholesterol. The reason why liver lipids did not continue to increase is unknown. The initial increase in liver lipids was just as rapid when the diet was low in fat as when it contained 10 per cent corn oil, but the total accumulation of TG was less on the low-fat diet. Neither diet contained cholesterol; its accumulation

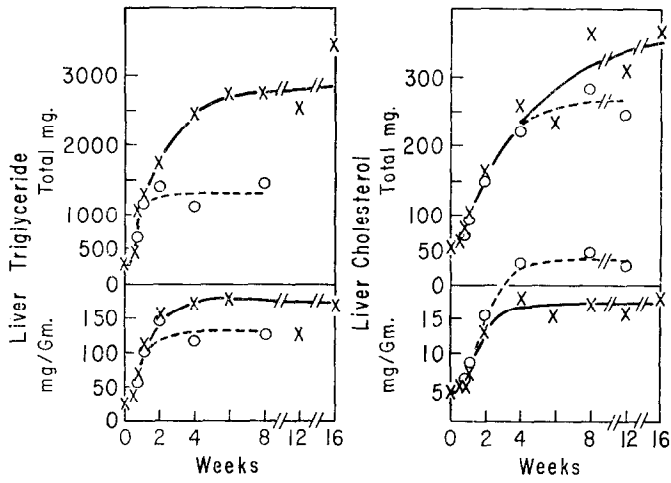

FIG. 3. Accumulation of liver TG and cholesterol as a result of adding 1 per cent orotic acid to a diet containing 10 per cent corn oil (21 per cent of the calories) (×) or a fat-free diet containing 5 per cent of the calories as ethyl linoleate (○). Male rats weighing 100 g were fed the indicated diets for 8–16 weeks; groups of five rats were sacrificed and analyzed at the indicated time intervals.

at a maximum rate of 10 mg/day in the liver represented *de novo* synthesis or a shift from other tissues to the liver.

A maximum of 300 mg TG/day accumulated in the liver during the rapid infiltration phase, and this was only about one-fifth of the daily intake. Serum lipids were decreased to low levels by the addition of orotic acid to either diet (20 mg/100 ml of TG; 15 mg/100 ml of cholesterol) within 4–6 days, and they remained at that level throughout the 16 weeks of the experiment. The feeding of orotic acid and the accumulation of liver lipids had little effect on growth (5 g/day between the fourth and eighth week). The addition of 0·25 per cent adenine to either orotic acid diet completely prevented all lipid changes in the liver and serum.[24,25]

TABLE 2. LIVER TRIGLYCERIDE AND CHOLESTEROL CONCENTRATIONS UNDER THE VARIOUS EXPERIMENTAL CONDITIONS*

	No treatment		+ T_4		+ CPIB	
Diet	TG	Chol.	TG	Chol.	TG	Chol.
Basal	12 ± 0·6†	4·8 ± 0·16†	9 ± 0·7	4·1 ± 0·17	5 ± 0·5	3·0 ± 0·24
DPTH	20 ± 2·2	6·4 ± 0·34	11 ± 0·9	5·3 ± 0·17	7 ± 0·9	3·4 ± 0·33
Imidazole	10 ± 0·8‡	4·3 ± 0·18‡	6 ± 0·5‡	4·0 ± 0·16‡	4 ± 0·4	2·7 ± 0·16
Orotic acid	124 ± 7·8	13·4 ± 0·49	63 ± 9·1	10·5 ± 0·57	10 ± 1·4	3·2 ± 0·24

* Values are given as milligrams/100 g of fresh liver. Mean ± S.E. for groups of 24 rats, except as noted below. Same rats as in Table 1. Liver weights averaged 3·1 per cent of the body weight (body wt. = 250–280 g at sacrifice) in the basal and imidazole groups ± T_4, 3·7 per cent with DPTH ± T_4, 4·6 per cent with orotic acid ± T_4, 4·1 per cent with DPTH + CPIB, and 4·5–4·8 per cent with the other CPIB groups.

† There were 72 rats in this group.

‡ There were 48 rats in this group.

The types of fatty acids accumulating in the liver were determined by gas chromatography. After either 4 or 8 weeks of orotic acid feeding with a 10 per cent corn oil diet, the major liver fatty acids were (as per cent of total fatty acids): palmitic, 23·7; palmitoleic, 3·4; stearic, 5·7; oleic, 23·5; linoleic, 37·0; and arachidonic, 6·5. The corresponding values on the low-fat diet were 28·0, 8·5, 6·5, 51·0, 3·5 and 2·3. The corn oil, which contained 53 per cent linoleic acid, gave rise to a high content of this fatty acid in the orotic acid fatty liver. When the diet was relatively fat free, the major fatty acid which accumulated in the liver was oleic acid. Therefore, dietary fat did contribute to an orotic acid fatty liver, but was not essential to the process.

The effects of the various additions to the diet on liver TG and cholesterol are given in Table 2. CPIB decreased these liver constituents under all of the experimental conditions tested. CPIB completely prevented the orotic acid fatty liver, and this effect was not mediated through T_4, since the latter had only a small effect. Either the CPIB reversed the orotic acid fatty liver by restoring the pre-β-lipoproteins (as

adenine does),[26] or it removed liver fat by by-passing the pre-β-lipoprotein block. These alternatives were resolved by serum electrophoresis.

Serum lipoproteins. Figure 4A shows the serum electrophoretic patterns for the rats in these studies. The lipoprotein pattern for these adult rats on the basal diet was characterized by a strong pre-β band and by a somewhat variable but generally weak β band. The administration of T_4, DPTH or imidazole had no obvious effect on this basal pattern (not shown), but small differences would have escaped detection. When CPIB was added to the diet, the characteristic feature of the serum lipoproteins was a strong β band (Fig. 4A), and this was unchanged by the simultaneous presence of DPTH or imidazole (not shown). Orotic acid usually eliminated the pre-β band completely and left a serum with only chylomicrons and α-lipoproteins. When Su 13437 or CPIB was also added to the orotic acid diet, a strong β band reappeared consistently; the intensity of this band varied with the dosage and the relative activities[32] of these drugs in preventing an orotic acid fatty liver (e.g. 0·01 % CPIB was too small to alter the orotic acid pattern). A small restoration of the pre-β band was also sometimes observed with these drugs. When T_4 was administered to orotic acid-fed rats, a pre-β (but no β) band often appeared. Since both the CPIB and T_4 had a small effect on the serum pre-β-lipoproteins, but only the CPIB had a major effect on the β-lipoproteins and the removal of liver fat, it can be concluded that the effect of CPIB was associated with the β-lipoproteins rather than the pre-β, and that the effect was not mediated through any T_4-like response.

The orotic acid block was completely reversed by adding adenine and a normal lipoprotein pattern was restored. DPTH acted like CPIB in affecting primarily the β-lipoproteins, while the addition of imidazole to the orotic acid diet had no effect on either the fatty liver or the restoration of either the pre-β or β bands (not shown separately, but similar to the orotic acid pattern).

CPIB vs. orotic acid fatty livers. In additional studies, rats were fed 1 per cent orotic acid with or without the various drugs for 2 weeks. The addition of 0·05 % CPIB to the orotic acid diet was just enough to keep the liver TG and cholesterol values close to the normal basal levels. Compound Su 13437 was more effective and decreased liver lipids to less than basal levels even in the presence of orotic acid. Imidazole was inactive in this test, but DPTH also completely prevented the development of an orotic acid fatty liver.

CPIB not only prevented the development of an orotic acid fatty liver, but it also caused a rapid removal of accumulated fat from such livers. Rats were fed orotic acid for 2 weeks to increase liver TG and cholesterol to approximately 90 and 12 mg/g respectively. CPIB, DPTH or adenine was then added to the diet, and groups of five rats each were sacrificed at intervals thereafter for the next 12 days. None of the drugs had any effect for the first 2 days, but by the fourth day the CPIB had decreased liver TG to 20 mg/g, and it remained between 10 and 17 mg/g thereafter. Liver cholesterol decreased more slowly and it was 8 days before the normal value of about 5 mg/g was reached. Adenine reduced liver TG by 50 per cent at 4 days and then more slowly to reach 20 mg/g by the twelfth day; liver cholesterol fell linearly after the second day to reach 6 mg/g by the twelfth day. DPTH caused a relatively slow linear decrease in liver TG between the second and twelfth days, but liver cholesterol did not begin to fall until after the seventh day of DPTH feeding.

Throughout this period of fat removal from the liver, serum TG and cholesterol were

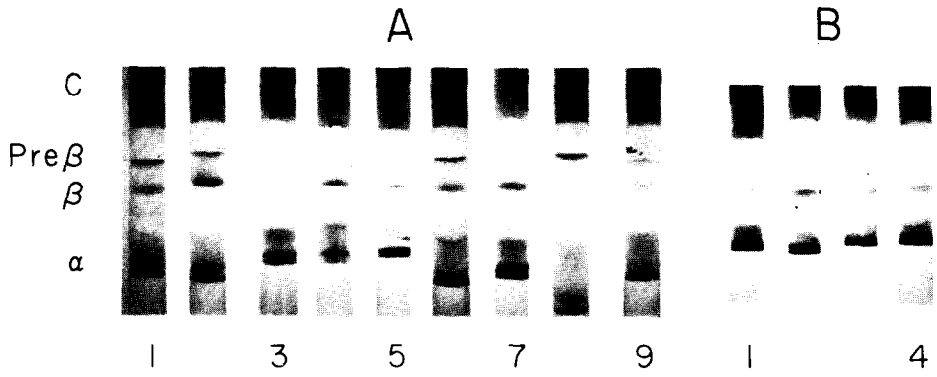

FIG. 4. A Serum lipoprotein patterns in rats fed orotic acid ± various drugs. Male rats (200 g; Miller) were fed the 1 per cent orotic acid diet plus the indicated amounts of each drug for 2 weeks. Differences in the height of the electrophoretic pattern as well as small differences in the location of the bands are due to variations in the filling of the electrophoresis tubes. (1) Basal; (2) basal + 0·3% CPIB; (3) 1% orotic acid; (4) orotic acid + 0·05% Su 13437; (5) orotic acid + 0·1% CPIB; (6) orotic acid + 0·25% adenine; (7) orotic acid + 0·06% DPTH (± 6 μg T_4/100 g of body weight/day); (8) orotic acid + 6 μg T_4; (9) 1% orotic acid for 8 weeks. B Serum lipoprotein patterns during the removal of fat from the liver by CPIB, DPTH or adenine. Male rats (200 g; Miller) were fed the 1 per cent orotic acid diet for 14 days. CPIB (0·3%), DPTH (0·06%) or adenine (0·25%) was then added to the orotic acid diet, and groups of rats were sacrificed at intervals thereafter. The results shown were obtained after 12 days, but similar results were obtained at 4 and 9 days. (1) Orotic acid; 2) orotic acid + CPIB; (3) orotic acid + DPTH; (4) orotic acid + adenine.

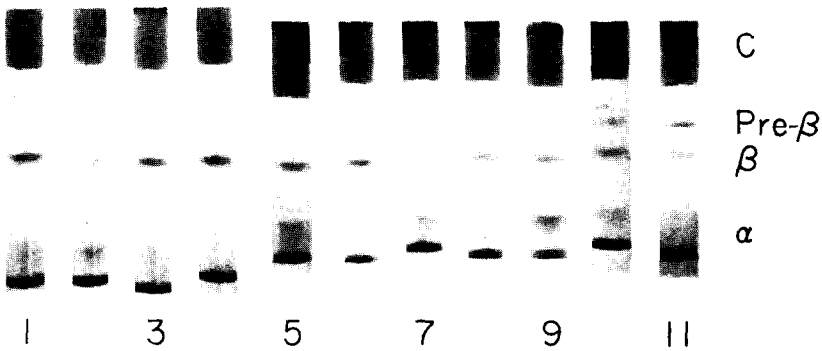

FIG. 5. Serum lipoprotein patterns of thyroidectomized rats. Weanling male Holtzman rats were thyroidectomized and fed chow until growth reached a plateau at 5 weeks. They were then fed the basal diet ± 1% orotic acid, 0·3% CPIB, 0·1% Su 13437 or 0·25% adenine for 2 weeks. Some were also injected subcutaneously with 1 μg T_4/100 g of body weight/day. (1) Basal; (2) orotic acid; (3) CPIB; (4) orotic acid + CPIB; (5) T_4; (6) T_4 + CPIB; (7) T_4 + orotic acid; (8) T_4 + orotic acid + CPIB; (9) T_4 + orotic acid + Su 13437; (10) T_4 + orotic acid + adenine; (11) untreated intact 200 g Holtzman rat on basal diet.

consistently low (approximately 20 and 16 mg/100 ml respectively) in rats fed CPIB. Serum TG increased to about 33 mg/100 ml with adenine and to 45 mg/100 ml with DPTH, while serum cholesterol increased to about 70 mg/100 ml with either drug (fourth to ninth day). After several days, the serum lipoprotein patterns in these rats became characteristic of the drug fed, and remained that way through the 12 days (Fig. 4B). The CPIB gave a strong β band; adenine restored both β and pre-β bands, and in these experiments the DPTH gave a pre-β as well as a β band.

Thyroidectomized rats. Weanling male rats (Holtzman) were thyroidectomized (Hormone Assay Lab.) and fed chow for 5 weeks until their growth reached a plateau at 150–160 g.[33] They were then fed various diets containing orotic acid plus the test substance for 2 weeks to see if these substances were active in the absence of the thyroid hormone. Orotic acid produced a fatty liver in thyroidectomized rats (TG = 42; cholesterol = 15 mg/g) and this was completely prevented by the simultaneous addition of CPIB, Su 13437, adenine or DPTH (TG = 3–8; cholesterol = 3–6 mg/g). Liver GPD was at the thyroidectomy level in all groups (3–6 units) except for orotic acid plus Su 13437 (9 units), and no increase in this enzyme was required for the prevention of an orotic acid fatty liver by CPIB.

The lipoprotein pattern for the Holtzman weanling rats[32] used in these studies was similar to that shown in Fig. 5 for the thyroidectomized rats fed the basal diet (no orotic acid), and it was characterized by a strong β and a weak or absent pre-β band. This differed from the pattern for the adult Miller rats used in the earlier studies (strong pre-β and weak β bands). Whether this was due to a difference in rat strains as well as to differences with age has not been determined. A pre-β-lipoprotein band was not produced in these thyroidectomized rats by feeding chow for 5 weeks or by the subsequent injection of 1 μg T_4/100 g of body weight/day for 2 additional weeks. Orotic acid diminished the intensity of the β band, and this was restored by adding CPIB (or Su 13437) to the orotic acid diet (Fig. 5). Hence the intensification of the β band by CPIB, as well as the prevention of an orotic acid fatty liver, took place in the absence of T_4 and at thyroidectomy levels of liver GPD. Adult Holtzman rats had a variable pre-β band, and the administration of adenine plus T_4 to the thyroidectomized rats (Fig. 5) was the only combination tried which also produced a pre-β band.

DISCUSSION

The effect of CPIB on fat metabolism was not mediated through its enhancement of T_4 effects in the liver. It had more effect on the handling of an exogenous fat load than did an amount of T_4 which produced the same liver GPD and malic enzyme changes. CPIB completely prevented or corrected an orotic acid fatty liver while T_4 had only a small effect. CPIB decreased the fasting blood lipids and produced a strong β-lipoprotein band in the electrophoretic pattern; T_4 did neither. The amount of CPIB which was required to prevent the orotic acid fatty liver and increase the serum β-lipoproteins was less than the amount required to produce major hyperthyroid effects in the liver.[32] Clearly CPIB affects lipid metabolism by a mechanism which does not respond to T_4.

The results show that β-lipoproteins can appear in the serum independently of pre-β-lipoproteins. Twenty-five per cent of the protein in the pre-β-fraction appears to be the same protein which makes up the β-lipoproteins,[34] and this could account for the small pre-β band sometimes seen in the serum of rats fed orotic acid plus CPIB.

It might also account for the decreased β-band produced in the thyroidectomized rats by orotic acid. It seems likely that the CPIB effect on serum β-lipoproteins was responsible for removing TG and cholesterol from the liver, since the two phenomena are both time and dose related.[32] CPIB also removes cholesterol from the aorta[35] and xanthomas.

Can the hypolipidemic effect of the drug also be attributed to an increased handling of lipids as β-lipoproteins? An increased concentration of β-lipoproteins in humans is usually associated with a relatively normal TG and an elevated cholesterol level, and seems to be due to a defect in removing β-lipoproteins from the plasma. In the normal dog, the β-lipoproteins do not contribute much to the total plasma TG or cholesterol.[36] If the increased β-lipoproteins are related to the hypolipidemic effect of the drug, this would seem to be possible only if it reflected a more rapid turnover of these plasma lipids,[37] possibly through handling of more of the lipid as β-lipoprotein.

Several interesting points emerged from the orotic acid feeding. Only 20 per cent or less of the dietary fat was trapped in the liver when the formation of pre-β lipoproteins was blocked. The other 80 per cent was either not absorbed or was taken up by other tissues without passing through the liver, secreted by the liver in a form other than β or pre-β-lipoprotein, or catabolized by the liver. In fact, dietary fat was not essential for the production of an orotic acid fatty liver. On a fat-free diet containing 5 per cent of the calories as ethyl linoleate, both the TG and cholesterol which accumulated in the liver were synthesized *in situ* or were transported to the liver from other tissues. A primary accumulation of liver cholesterol might be the cause of a secondary TG accumulation, but TG was removed more rapidly than cholesterol in a reversal of the orotic acid fatty liver by any of the drugs.

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