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HYPERALPHALIPOPROTEINAEMIC ACTIVITY OF BRL 26314—I

ENHANCED CHOLESTEROL TURNOVER IN RATS

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Abstract—Administration of BRL 26314 [*N*-(4-chlorobenzyl)-L-phenylalanine] raises circulating high-density lipoprotein (HDL) cholesterol and lowers total triglyceride levels in rats whether maintained on stock or semi-synthetic diets. HDL is also elevated by BRL 26314 in hypothyroid rats and in rats with pre-existing hyperlipidaemia where aortic total cholesterol concentration is decreased. BRL 26314 promotes the excretion of a dose of radiolabelled cholesterol as faecal sterols and bile acids, and decreases the extent of cholesterol-radiolabelling in tissue pools, particularly the aorta and adipose tissue. The increase in cholesterol and bile acid (cholic acid) turnover distinguishes BRL 26314 from a cholestatic agent such as 1-naphthyl isothiocyanate where a superficially similar change in HDL concentration disguises an impaired cholesterol transport. BRL 26314 is not a general protein inducer but part of the mechanism of action may involve enhancement of white adipose tissue lipoprotein lipase activity.

Recent findings on the contribution of serum lipids to the development of arteriosclerosis emphasize the role of high-density lipoprotein (HDL) as a negative risk factor in addition to the function of low-density lipoprotein (LDL) and possibly other apoprotein B-containing lipoproteins as positive risk factors. Since the rediscovery of this putative role for HDL [1], a variety of epidemiological evidence has correlated the circulating level of HDL cholesterol with the incidence of coronary heart disease [2, 3], cerebrovascular disease [4] and with total mortality [5].

Hypolipidaemic agents studied in the clinic have been claimed to elevate HDL, the evidence perhaps being strongest for nicotinic acid [6], but these standard drugs have multiple actions on lipid metabolism and it is not possible to determine to what extent the modest amelioration of coronary heart disease [7] can be attributed to changes in the level of HDL rather than LDL or very-low-density lipoprotein (VLDL). It is generally agreed that the drugs available at present do not satisfy the criteria of consistent efficacy, safety and palatability so that there is potential for new agents, particularly if cholesterol turnover can be promoted by novel mechanisms of action.

The relevance of lipoprotein changes in rats to the prediction of the clinical response has been much discussed [8, 9]. In general, the lipoproteins of laboratory mammals differ chemically from those of man to only a small degree [10], presumably because of the common need for an efficient means of lipid transport. The normolipidaemic rat, however, may be a relatively inappropriate model for testing either hypobetalipoproteinaemic or hyperalphalipoproteinaemic compounds [9] unless some attention is devoted to measuring lipid transport rather than concentration. There has been little detailed examination of specific lipoprotein changes in rats fed atherogenic diets [11] but recently we developed a diet inducing moderate elevations of LDL and VLDL without depletion of HDL or accumulation of abnormal lipoproteins [12].

BRL 26314 [*N*-(4-chlorobenzyl)-L-phenylalanine] (Fig. 1) belongs to a novel series of compounds that elevate serum total HDL cholesterol in rats [13]. The present work describes how the effect of BRL 26314 on HDL metabolism in both normolipidaemic and hyperlipidaemic rats is associated with enhanced cholesterol centripetal transport and faecal excretion of sterols and bile acids.

MATERIALS AND METHODS

Animals, diets and treatment. Male Sprague–Dawley rats (140–160 g) were obtained and maintained on a stock diet or on a sucrose-based diet as described previously [12]. Inclusion of 0.25% cholesterol plus 0.05% cholic acid in this semi-synthetic diet induced an exacerbated hyperbetalipoproteinaemia by comparison with an equivalent supplement to the stock diet. Dietary cholesterol and sucrose, when combined in these proportions, may interact to maintain

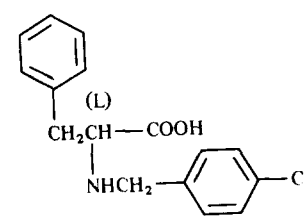

Fig. 1. Structure of BRL 26314.

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high VLDL secretion rates, thus allowing relatively high LDL synthesis and partially alleviating feedback regulation of cholesterogenesis (R. Fears, unpublished results).

Rats were allocated to experimental groups (8 rats/group, 4 rats/cage) so that the mean body weight of each group was similar. Where applicable, body weight was recorded throughout the experimental period. BRL 26314 was administered as a supplement to the diet (0.1% w/w) for 7 days. Rats were killed between 1000 and 1100 hr (daylight-dependent light cycle).

Measurement of serum lipids and lipoproteins and tissue lipids. Lipids were measured using a Technicon AutoAnalyser II with enzymatic methods for cholesterol [14] and triglycerides [15], the latter adapted for fluorimetry. HDL, LDL and VLDL were separated by chemical precipitation methods as described previously [12] or by preparative ultracentrifugation [16]. Washed lipoproteins were dialysed against 100 vols. 0.15 M NaCl–0.001 M EDTA, pH 7.0, for 24 hr and purity was checked by polyacrylamide gel electrophoresis. Where applicable, HDL was lyophilized and delipidated using ethanol–ether (3:1, v/v).

Lipogenesis. The synthesis of digitonin-precipitable sterols and fatty acid *in vivo* was measured 1 hr after an intraperitoneal (i.p.) injection of ^3H_2O and extraction of tissue lipids with petroleum ether as described previously [17]. The synthesis of triglycerides, diglycerides and phospholipids in liver slices *ex vivo* was also measured by methods described previously [18] using [1-^{14}C]glycerol as substrate with extraction of lipids using chloroform–methanol and isolation by thin-layer chromatography (TLC).

Lipid turnover. The activity of lipoprotein lipase in an alkaline extract of epididymal adipose tissue was measured with a synthetic triolein substrate as described previously [18]. The same method was used for the assay of enzyme activity in interscapular brown adipose tissue.

The activity of hepatic microsomal cholesterol 7α-hydroxylase was measured as described elsewhere using radiolabelled cholesterol substrate solubilized in Tween 80 and isolating the product by TLC [19].

For the measurement of cholesterol turnover, rats were injected i.p. with [1α,2α(n)-^{3}H]cholesterol (10 μCi/100 g body wt) in ethanol–0.9% NaCl (3:47, v/v), 0.2 ml/100 g body wt, and faeces were collected from each cage of 4 rats, daily, for 8 days. At the end of the experiment, tissues were taken for total combustion using an Intertechnique 'Oxymat' tissue oxidizer and thence for scintillation counting. The daily faecal output from each cage was dried at 80° for 2 hr, ground to a fine powder and weighed. For the extraction of sterols, 10 ml 15% KOH (w/v in 95% ethanol) was added to 500 mg of sample and the mixture autoclaved at 104 kPa for 3 hr. Radiolabelled sterols were extracted with 3 × 10 ml light petroleum (b.p. 40–60°) and an aliquot was counted. Total radioactivity in samples of dried faeces was measured after combustion using the tissue oxidizer and radioactivity as bile acids was calculated by subtraction of sterols from total radioactivity.

Bile duct cannulation. Cannulation was performed using rats (200–250 g) anaesthetized with pentobarbitone (60 mg/kg body wt) according to the procedure described by Wade [20]. For the measurement of bile acids, bile samples were deconjugated using choloylglycine hydrolase and the free bile acids were extracted into diethyl ether. For the separation of individual bile acids by gas chromatography, trifluoroacetylated methyl esters were prepared [21], with 12-ketolithocholic acid as internal standard, and analysed by capillary column gas chromatography using a Fractovap 4160 apparatus (Carlo Erba, Milan, Italy) with a flame ionization detector and a fused silica capillary column (25 × 0.2 mm) coated with 0.12 μM CP^{tm} Sil 5 (Chrompak UK, London, U.K.) with N_2 as carrier gas (flow rate: 1 ml/min). Injection and detector temperatures were 280°, oven temperature was 270° and split ratio was approximately 10:1. Retention times (min) were: lithocholic acid, 6.5; deoxycholic acid, 7.5; chenodeoxycholic acid, 8.8; 12-ketolithocholic acid, 9.2; cholic acid, 9.7. Total bile acids were also measured by using 3α-hydroxysteroid dehydrogenase [22].

Other measurements. Methods for the isolation of hepatic microsomes and for the determination of microsomal cytochrome P-450 and protein have been reported elsewhere [19]. Aminopyrine *N*-demethylase activity was measured according to Holtzman *et al.* [23] with formaldehyde production measured by the method of Nash [24].

Chemicals. [4-^{14}C]Cholesterol (sp. radioact. 54–57 mCi/mmole), [1α,2α(n)-^{3}H]cholesterol (sp. radioact. 50 Ci/mmole), ^3H_2O (sp. radioact. 18 Ci/mmole) and [1(3)-^{14}C]glycerol (sp. radioact. 50.8 mCi/mmole) were obtained from Amersham International (Amersham, U.K.). 1-Naphthyl isothiocyanate (ANIT) was obtained from Aldrich Chemical Co. Ltd. (Dorset, U.K.) and sodium phenobarbitone from Evans Medical Ltd. (Speke, Liverpool, U.K.). Choloylglycine hydrolase was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). BRL 26314 was prepared by the method described previously [13]. The majority of the experiments described used the L-isomer although the DL-racemic mixture has been investigated and shows similar activity.

RESULTS

Hyperalphalipoproteinaemic activity of BRL 26314

Inclusion of BRL 26314 in the diet for 7 days raised the serum concentration of HDL cholesterol measured after precipitation of LDL and VLDL, whether rats were maintained on the stock diet or the sucrose-rich cholesterol-supplemented diet (Table 1). Total serum cholesterol was also increased but the total triglyceride concentration was decreased (statistical differences being more difficult to demonstrate in the hyperlipidaemic rats because of greater variance).

BRL 26314 did not affect the concentration of lipids in the liver nor was there any indication of gross toxicity as determined by gain in body weight or liver weight (Table 1) or the weights of kidneys and spleen, the serum activities of glutamate-pyruvate transaminase, glutamate-oxaloacetate transaminase, alkaline phosphatase, the concentration of

Table 1. Comparision of effects of BRL 26314 (100 mg/kg for 7 days) in rats receiving stock and semi-synthetic diets

	Stock diet		Semi-synthetic diet	
	Control	BRL 26314	Control	BRL 26314
Gain in body weight (g)	46 ± 3.1	48 ± 2.9	48 ± 1.2	45 ± 2.0
Liver wt (g/100 g body wt)	5.0 ± 0.2	5.3 ± 0.3	5.7 ± 0.4	6.0 ± 0.2
Serum lipid (mg/100 ml)				
Cholesterol	65 ± 1.7	73 ± 2.1*	108 ± 3.3	125 ± 7.7
Triglyceride	85 ± 6.1	53 ± 4.0*	181 ± 2.6	126 ± 9.6
HDL cholesterol	52 ± 1.5	59 ± 1.8*	43 ± 2.0	66 ± 3.1**
LDL cholesterol	—	—	22 ± 3.0	32 ± 4.7
VLDL cholesterol	—	—	40 ± 2.1	28 ± 3.2**
Serum phospholipid (μmole/100 ml)	—	—	164 ± 8.8	192 ± 12.3
Liver lipid (mg/g)†				
Total	42	40	65	61
Cholesterol	2.3	2.2	12.4	10.6
Triglyceride	5.1	5.0	14.0	11.5
Liver lipid synthesis (μg/hr per g wet wt)				
Sterols	43 ± 7.1	51 ± 5.0	24 ± 4.7	31 ± 5.5
Fatty acid	80 ± 10	95 ± 15	451 ± 43	525 ± 49

Each rat was injected intraperitoneally with ^3H_2O (1.5 mCi/100 g body wt) 1 hr prior to killing. Results are the means ±S.E.M. for eight analyses; each analysis being of tissue from a single rat.

* Indicates a significant difference from a comparable control group ($P < 0.05$).

** Indicates a significant difference from a comparable control group ($P < 0.01$).

† Analysis performed on tissue pooled from eight rats.

urea N, or the activity of hepatic peroxisomal catalase (results not shown).

Slight increases in hepatic cholesterogenesis, measured *in vivo*, did not achieve statistical significance (Table 1) although the release of newly-synthetized sterol into the circulation was also modestly enhanced (1 hr after administration of ^3H_2O, serum-sterol radiolabelling was 60 dpm/ml in control, stock-fed rats, increasing to 80 dpm/ml in rats treated with BRL 26314). BRL 26314 also had no significant effect on sterol synthesis in either small intestine (62 ± 3.8 by comparison with the control level of 75 ± 3.2 μg/hr per g wet wt) or kidney (23 ± 2.9 by comparison with the control level of 34 ± 4.8 μg/hr per g wet wt) in rats fed on the semi-synthetic diet. In other experiments, with either diet, liver slices were prepared and incubated *ex vivo* with [1-^{14}C]glycerol but BRL 26314 did not affect the synthesis of any of the major classes of glycerolipids. For example, using the semi-synthetic diet, glycerol incorporation (nmole/hr per g wet wt) into phospholipids was 65 ± 5.8 (control, 62 ± 2.3); diglycerides, 20 ± 1.4 (control, 23 ± 2.9); triglycerides, 134 ± 6.2 (control, 132 ± 6.9). Similarly, there was no effect on the synthesis of individual classes of phospholipids (choline, ethanolamine, serine). BRL 26314 administration at 100 mg/kg for 7 days to rats maintained on the semi-synthetic diet did affect the activity of lipoprotein lipase measured *ex vivo*. Epididymal fat pad (white adipose tissue)

Table 2. Comparison of lipoprotein assays by polyanion-precipitation and by ultracentrifugation

	Control (semi-synthetic diet)	BRL 26314 (100 mg/kg for 7 days)
Serum cholesterol (mg/100 ml)	110 ± 4.5	136 ± 3.5**
Heparin/Mn^{2+} precipitation		
HDL cholesterol (mg/100 ml)	52 ± 3.0	68 ± 4.1**
LDL cholesterol (mg/100 ml)	40 ± 2.5	50 ± 2.1*
Ultracentifugation†		
Cholesterol (mg/100 ml)		
HDL (*d* 1.063 – 1.210)	50	75
LDL + IDL (*d* 1.006 – 1.063)	48	45
Phospholipid (μmol/100 ml)		
HDL (*d* 1.063 – 1.210)	55	70
LDL (*d* 1.006 – 1.063)	45	50

Results are the means ±S.E.M. for eight analyses; each analysis being of tissue from a single rat.

* Indicates a significant difference from the control group ($P < 0.05$).

** Indicates a significant difference from the control group ($P < 0.01$).

† Mean of two analyses; each analysis being of serum pooled from four rats.

activity increased from a control level of 69 ± 3.1 to 100 ± 5.9 nmole fatty acid/hr per mg protein (n = 8, $P < 0.01$) but interscapular fat pad (brown adipose tissue) activity was unchanged (17 ±1.6 by comparison with the control level of 16 ± 1.3 nmole fatty acid/hr per mg protein).

A variable increase in serum LDL cholesterol concentration was observed in some experiments. However, the LDL fraction as measured by heparin/Mn^{2+} polyanion-precipitation includes HDLc which tends to accumulate in cholesterol-fed rats, although minimized by using the present semi-synthetic diet [12]. When serum samples from BRL 26314-treated rats were fractionated by polyacrylamide gel electrophoresis there was no increased staining in the band with β-mobility (LDL), so further information was obtained by differential flotation after high-speed centrifugation. In an experiment where both HDL cholesterol and 'LDL' cholesterol, as measured by polyanion-precipitation, were significantly increased in concentration, only HDL was increased when measured after isolation by ultracentrifugation (Table 2)—this fraction includes any HDLc present in the circulation [25]. It should be noted that in the density range used to isolate LDL (1.006–1.063), HDL_1 may also be present [26]. There is no evidence (Table 2) that HDL_1, a product of chylomicron catabolism [26], is increased by BRL 26314. When HDL (d 1.063–1.210) was delipidated and the apoproteins separated by polyacrylamide gel electrophoresis, the major band was $apoA_I$ (mol. wt 28,000) with other bands identified as $apoA_{IV}$ (49,000), arginine-rich peptide (35,000) and two apoC peptides (10,000; 11–12,000) in agreement with the results of other workers [27]. The relative intensity of protein-staining amongst the bands was similar in the control and drug-treated groups.

To provide a closer approximation to the possible clinical requirements, BRL 26314 was also tested in rats with pre-existing hypercholesterolaemia. When rats were maintained on the semi-synthetic diet for 14 days prior to commencement of drug treatment, control serum cholesterol reached a maximum although the concentration of serum triglycerides continued to rise subsequently (Table 3). Under these conditions, BRL 26314, when included in the diet for a further 7 days, again induced hyperalphalipoproteinaemia and hypotriglyceridaemia. BRL 26314 was also administered to a group of eight older rats that had been maintained on the semi-synthetic diet for 30 weeks. HDL cholesterol was again elevated (77 ± 4.3 mg/100 ml compared with 67 ± 2.7 mg/100 ml for comparable control rats) in association with a significant reduction in aortic total cholesterol (1.37 ± 0.05 mg per g wet wt compared with the control concentration 1.56 ± 0.04 mg per g, $P < 0.05$). We also combined the semi-synthetic diet with induction of thyroid damage (using 2-thiouracil, 0.2 g/100 g diet for 7 days) to produce exacerbated hyperbetalipoproteinaemia. Using these experimental conditions, BRL 26314 also raised the concentration of HDL cholesterol, even though basal levels were already high, as in human studies [28] (90 ± 5.5 mg/100 ml compared with 70 ± 2.0 mg/100 ml in hypothyroid control rats, $n = 8$, $P < 0.01$), again without an effect on the other lipoprotein classes (or on liver lipids).

Bile acid and sterol metabolism

To determine whether the HDL-elevating action of BRL 26314 was related to an accelerated centripetal sterol transport and catabolism, the turnover of radiolabelled cholesterol was studied. For this purpose we used normal rats given a stock diet so that the results were not complicated by the degree of individual responsiveness to dietary cholesterol. During the course of the 7 day experimental period, BRL 26314 increased the proportion of the radiolabelled cholesterol i.p. dose recovered in the faeces as bile acids to 59% (control recovery, 46%) and as sterols to 3.5% (control recovery, 1.8%). Statistical analysis of this approximate 35% increase in excretion is not possible because pooled faeces were collected from two cages of four rats per experimental group. At the end of the experiment, serum HDL

Table 3. Hyperalphalipoproteinaemic activity of BRL 26314 in rats with pre-existing hyperlipidaemia

	Control (semi-synthetic diet)	BRL 26314 (100 mg/kg for 7 days)
Gain in body weight (g)	41 ± 2.8	40 ± 1.5
Serum lipids (mg/100 ml)		
At start (14 days on diet)		
Cholesterol	117 ± 5.8	120 ± 5.5
Triglyceride	101 ± 10.8	112 ± 19.1
HDL cholesterol†	42	41
After 7 days experiment		
Cholesterol	117 ± 3.0	127 ± 4.2
Triglyceride	209 ± 22	111 ± 9.1*
HDL cholesterol	43 ± 2.9	64 ± 2.8*
LDL cholesterol	15 ± 1.1	20 ± 6.9
VLDL cholesterol	59 ± 2.9	51 ± 4.4

Results are the mean ±S.E.M. for eight analyses; each analysis being of tissue from a single rat.

* Indicates a significant difference from the control group ($P < 0.001$).

† Mean of two analyses; each analysis being of serum pooled from four rats.

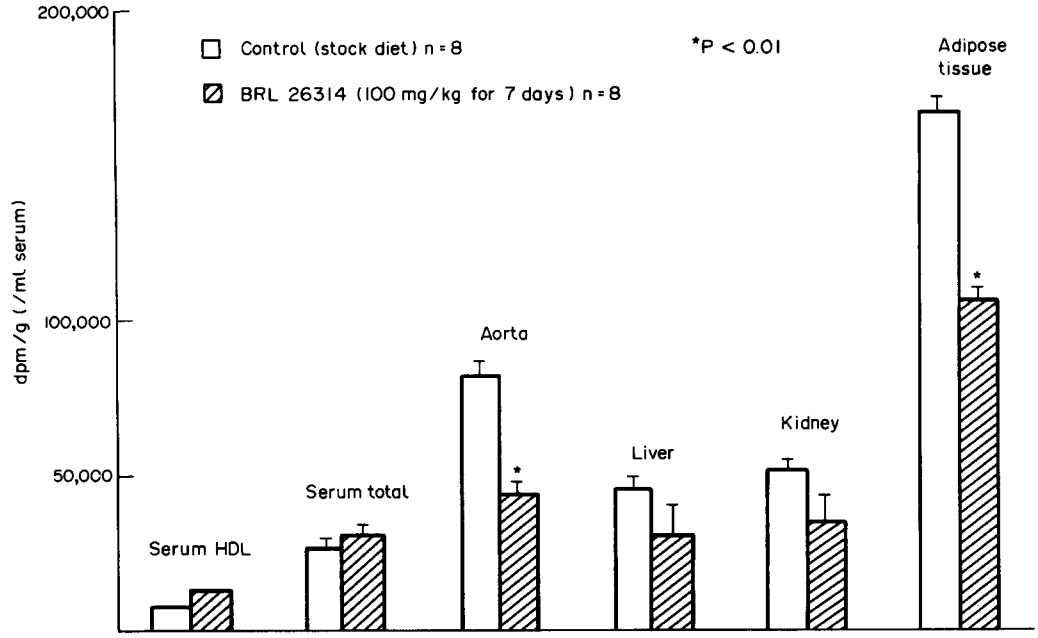

Fig. 2. Effect of BRL 26314 on tissue radiolabelled cholesterol remaining after 7 days.

cholesterol was increased to 59 ± 5.1 mg/100 ml in the BRL 26314-treated group by comparison with the control concentration, 45 ± 2.0 mg/100 ml ($P < 0.02$) and this change was accompanied by a similar relative increase in HDL radiolabelled cholesterol (Fig. 2) which accounted for the increment in serum total radiolabelled cholesterol. The increased faecal excretion of radiolabelled cholesterol as sterols and bile acids was also associated with decreased cholesterol radiolabelling in liver, kidney, aorta and epididymal fat pad with the changes at the latter two sites (44 and 40%, respectively) achieving statistical significance.

The promotion of bile acid excretion by BRL 26314 was studied in further detail in hyperlipidaemic rats after bile duct cannulation (Table 4). BRL 26314 did not affect bile flow and the small increment in cholesterol secretion was not statistically significant. However, the hourly secretion of total bile acid, as measured by using 3-hydroxysteroid dehydrogenase, was significantly increased and this change could be attributed specifically, using gas chromatography, to enhanced cholic acid flux. The biliary concentrations of chenodeoxycholic and deoxycholic acids were not affected by BRL 26314 although, as expected, the control levels were very high by comparison with age-matched rats maintained on the stock diet (0.25 ± 0.07 mg/hr chenodeoxycholic acid; 0.07 ± 0.03 mg/hr deoxycholic acid). Secretion of hyodeoxycholic acid was less than 0.1 mg/hr in both control

Table 4. BRL 26314 and bile composition

	Control (semi-synthetic diet) (9)	BRL 26314 (100 mg/kg for 7 days) (11)
Serum lipid (mg/100 ml)		
Cholesterol	119 ± 5	141 ± 5**
HDL cholesterol	41 ± 2	57 ± 6*
Bile flow (ml/hr per 100 g body weight)	0.34 ± 0.02	0.37 ± 0.02
Biliary secretion (mg/hr)		
Cholesterol	0.108 ± 0.010	0.123 ± 0.011
Total bile acids	3.67 ± 0.29	4.92 ± 0.43*
Cholic acid	2.65 ± 0.18	3.82 ± 0.47*
Chenodeoxycholic acid	0.44 ± 0.05	0.39 ± 0.08
Deoxycholic acid	0.54 ± 0.07	0.48 ± 0.08

At the end of the experimental period (1000 hr on day 8) rats were anaesthetized with sodium pentobarbitone (60 mg/kg body wt) and bile was collected for 2 hr. Results are the means ± S.E.M. for the number of rats indicated in parentheses.

* Indicates a significant difference from the control group ($P < 0.05$).

** Indicates a significant difference from the control group ($P < 0.01$).

Table 5. Comparison of BRL 26314 and ANIT

	Control (semi-synthetic diet)	ANIT (25 mg/kg for 7 days)	BRL 26314 (100 mg/kg for 7 days)
Serum lipid (mg/100 ml)			
Cholesterol	142 ± 13	135 ± 9	137 ± 4
Triglyceride	390 ± 41	329 ± 51	238 ± 34*
HDL cholesterol	34 ± 7	46 ± 6*	49 ± 5*
Hepatic microsomal metabolism			
Cholesterol 7α-hydroxylase (pmole/min per mg protein)	22.4 ± 3.6	16.3 ± 0.8	22.7 ± 4.1
Aminopyrine *N*-demethylase (nmole/min per mg protein)	2.9 ± 0.2	1.3 ± 0.1†	2.5 ± 0.2
Cytochrome P-450 (nmole/mg protein)	0.47 ± 0.04	0.44 ± 0.04	0.57 ± 0.04

Results are means ± S.E.M. for eight analyses; each analysis being of tissue from a single rat.
* Indicates a significant difference from the control group ($P < 0.05$).
† Indicates a significant difference from the control group ($P < 0.001$).

and BRL 26314-treated groups, and the secretion of hyocholic and lithocholic acids was each less than 0.2 mg/hr.

Comparison of the metabolic effects of BRL 26314 with the response to ANIT-induced cholestasis

It seemed likely, therefore, that the rise in HDL cholesterol observed after BRL 26314 administration was associated with increased cholesterol turnover, but it was considered useful to confirm that the metabolic response to BRL 26314 was dissimilar to the response found during cholestasis. It is known that most types of liver disease are characterized by low circulating HDL but cirrhosis leads to increased HDL_2 [29], and an abnormal HDL species, perhaps modified HDL_1, has been found to accumulate in cholestasis [30] and this would presumably be associated with attenuated cholesterol turnover. BRL 26314 at 100 mg/kg was compared directly with the cholestatic agent ANIT at 25 mg/kg, a level previously observed not to be grossly toxic (R. Fears, unpublished results). Both compounds appeared to increase serum HDL cholesterol concentration (Table 5) although ANIT was not hypotriglyceridaemic. However, serum lipoproteins were also separated using polyacrylamide gel electrophoresis and an additional lipid-staining band with β_1-mobility was observed in the samples from the rats treated with ANIT, analogous to the abnormal HDL identified in cholestatic plasma by other workers [30]. ANIT and BRL 26314 were also different as regards their effects on hepatic microsomal metabolism. Only ANIT decreased the activity of aminopyrine *N*-demethylase (Table 5) in association with a decrease in microsomal total protein (results not shown) and a slight decrement in cytochrome P-450 concentration. ANIT also tended to decrease microsomal 7α-hydroxylase activity and although this change did not attain statistical significance when expressed per mg microsomal protein, the activity per g liver was significantly decreased (381 ± 20 pmole/min by comparison with the control level, 587 ± 40 pmole/min, $P < 0.01$). This reduction can be attributed to feedback inhibition by the accumulating serum and tissue bile acids [31]. Neither compound affected either mitochondrial or microsomal side-chain oxidation of [26-^{14}C]cholesterol (results not shown).

The effect of ANIT on cholesterol turnover in stock-fed rats was also determined (Table 6, Fig. 3). At 25 mg/kg, ANIT significantly increased the concentration of cholesterol carried by all lipoprotein fractions although it should again be emphasized that these lipoproteins may be abnormal in composition. By contrast with the previous results for BRL 26314, ANIT decreased the excretion of cho-

Table 6. Effect of ANIT on cholesterol turnover

	Control (stock diet)	ANIT (25 mg/kg for 7 days)
Serum cholesterol (mg/100 ml)		
Total	62 ± 1.1	112 ± 8.9*
HDL	44 ± 1.2	74 ± 3.7*
LDL	8 ± 1.6	21 ± 3.4*
VLDL	9 ± 0.8	17 ± 0.9*
% Dose recovered in faeces/rat		
Bile acids	53	35
Sterols	1.3	0.4

Results are means ± S.E.M. for eight analyses; each analysis being of tissue from a single rat.
* Indicates a significant difference from the control group ($P < 0.001$).

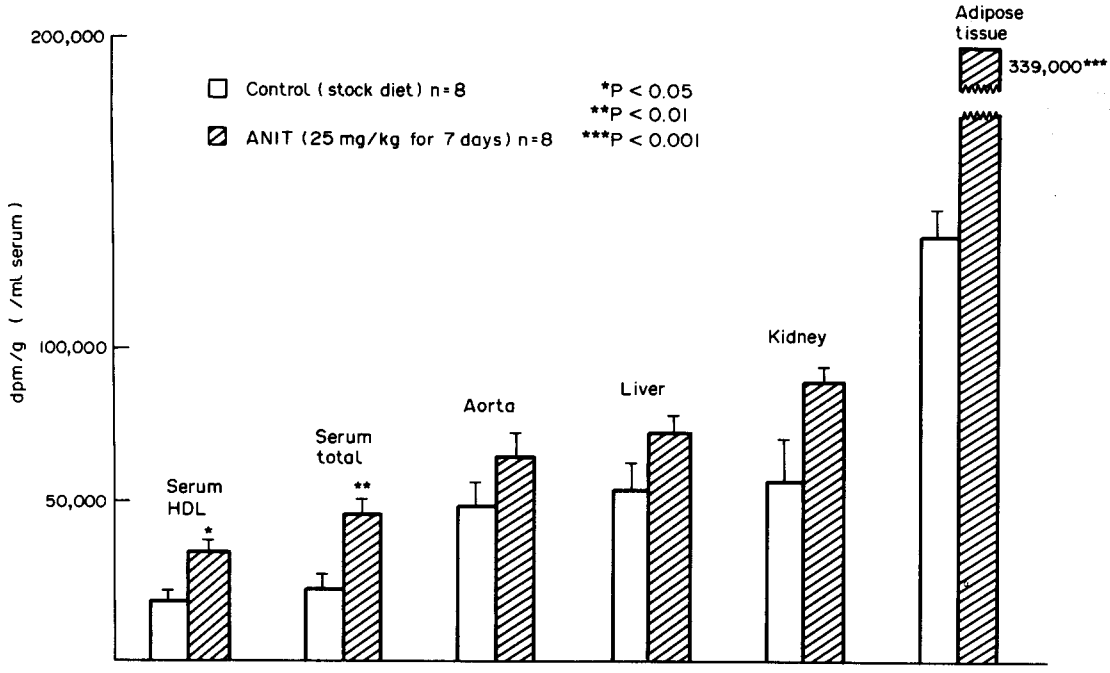

Fig. 3. Effect of ANIT on tissue radiolabelled cholesterol remaining after 7 days.

lesterol as sterols and bile acids by approximately 40%. Radiolabelling of HDL cholesterol was significantly increased (Fig. 3) although this did not account for the entire increase observed in serum total cholesterol radioactivity. By contrast with the results for BRL 26314 (Fig. 2), ANIT increased the amount of radiolabelled cholesterol remaining in the aorta, liver, kidney and adipose tissue although only the latter difference (+250%) achieved statistical significance. ANIT at a dose level of 10 mg/kg (results not shown) also elevated serum lipoprotein levels and increased the amount of radiolabelled cholesterol remaining in aorta, kidney and adipose tissue in association with decreased faecal excretion of sterols and bile acids (−30%).

Response to phenobarbitone

Although BRL 26314 does not act as a general inducer of protein metabolism (Table 5), it was considered of interest to determine, using our experimental conditions, the consequences of inducing cytochrome P-450 and drug-metabolizing enzymes. When administered i.p. at 50 mg/kg for 7 days, sodium phenobarbitone, unlike BRL 26314, increased liver weight, liver lipid, microsomal protein and aminopyrine *N*-demethylase activity (Table 7). The modest increase in serum HDL cholesterol concentration did not achieve statistical significance in agreement with results from comparable chronic studies [32].

DISCUSSION

The circulating concentration of HDL seems to be affected only modestly by the programmes of conventional risk factor intervention [33] and so it may be considered desirable to identify compounds to elevate HDL turnover therapeutically. In addition to the commercially available hypolipidaemics that are claimed to affect HDL, there are a number of other pharmacologically important compounds which have been observed to raise HDL cholesterol in man, e.g. phenytoin [34], cyclofenil [35], terbutaline [36] and Rowachol [37]. However, apart from considerations of specificity and potency, it has yet to be established that a pharmocological increase in serum HDL cholesterol is associated with increased centripetal cholesterol transport and reduced risk of atherosclerosis [38]. In the present studies, using rats, BRL 26314 raised the serum concentration of cholesterol carried by HDL, and perhaps also by HDLc, in association with the reduction of aortic total cholesterol content and enhanced biliary secretion of cholic acid. BRL 26314 also accelerated the excretion of a dose of radiolabelled cholesterol as bile acids and sterols thereby depleting the rapidly-turning-over tissue pools of cholesterol, particularly in the aorta and in adipose tissue.

It should be emphasized that the response to BRL 26314 is not characteristic of enzyme induction, i.e. there is no increase in liver weight, phospholipid biosynthesis, microsomal protein or drug-metabolizing enzymes. Furthermore, standard protein-inducing agents such as phenobarbitone (Table 7) and phenytoin (R. Fears, unpublished results) have relatively little effect on the concentration of HDL cholesterol under the present experimental conditions, in contrast with the results from certain other studies [39]. We have obtained no evidence to show that the HDL induced by BRL 26314 is abnormal in composition and the general metabolic response is also dissimilar to that observed for the toxins tetrachlorodibenzodioxin [40] and polychlorinated biphenyls

Table 7. Effect of phenobarbitone on lipid and drug metabolism

	Control (stock diet)	Phenobarbitone (50 mg/kg i.p. for 7 days)
Liver weight (g/100 g body weight)	4.4 ± 0.2	5.3 ± 0.3*
Liver total lipids†	47.9	57.8
Serum cholesterol (mg/100 ml)		
Total	53 ± 3.1	56 ± 2.6
HDL	38 ± 2.0	44 ± 2.8
Hepatic microsomal protein (mg/g liver)	16.3 ± 0.8	21.4 ± 1.0*
Hepatic microsomal aminopyrine *N*-demethylase (nmole/min per mg protein)	4.8 ± 0.6	6.2 ± 0.3*

Results are the means ±S.E.M. for eight analyses; each analysis being of tissue from a single rat.

* Indicates a significant difference from the control group ($P < 0.05$).

† Analysis performed on tissue pooled from eight rats.

[41]. In particular, BRL 26314 is not cholestatic and the net effect is dissimilar from the response to ANIT where a superficially similar rise in HDL, as measured after heparin/Mn^{2+} precipitation, disguises an impaired turnover of cholesterol.

There have been various mechanisms postulated whereby HDL cholesterol concentration might be raised and we have attempted to investigate in detail certain aspects to account for the mode of action of BRL 26314. Certain key steps in the metabolism of HDL can be adumbrated as follows. Nascent HDL, rich in phospholipid, apoproteins A, E and free cholesterol, is secreted by the liver and small intestine. Subsequent remodelling in the circulation involves both the transfer of lipids and apoproteins A and C from triglyceride-rich lipoproteins (during their catabolism by lipoprotein lipase) and also the uptake of cholesterol from extrahepatic tissues. Reorganization of the nascent bilayer into mature, spheroidal HDL also involves the conversion of surface-free cholesterol to hydrophobic cholesteryl esters by lecithin-cholesterol acyltransferase. Donation of cholesterol and phospholipid back to the liver with possible return of lipid-depleted HDL to the circulation may involve heparin-releasable hepatic lipase. It is not yet generally established which, if any, of these metabolic steps is rate-limiting. Our information on BRL 26314 is as follows.

We do not know whether BRL 26314 affects the synthesis or secretion of apoprotein A although it is not a general protein inducer. The compound does not increase hepatic phospholipid synthesis although the slight increase observed in synthesis and release of hepatic cholesterol might be sufficient to support enhanced nascent HDL production. The activity of lipoprotein lipase in white adipose tissue is significantly increased by BRL 26314 and this effect probably accounts for the hypotriglyceridaemic activity and may well be central to the enhanced production of HDL. An increase in lipoprotein lipase activity could also explain the variable rise observed in LDL concentration (a product of the reaction) although we believe that, at least in part, any rise in 'LDL' can be attributed to the accumulation of HDLc. However, other mechanisms must also be important in the response to BRL 26314 because the compound is also active in rats given Triton WR 1339 (R. Fears, unpublished results) where lipoprotein lipase activity is impaired and because the compound also stimulates cholesterol uptake from extrahepatic tissues, particularly the aorta.

Whatever the primary mechanism for elevating HDL biosynthesis, and whether or not the variable rise in LDL is genuine, the hyperalphalipoproteinaemic state promotes cholesterol return to the liver for excretion both as sterols and bile acids, the latter predominating. Although HDL-free cholesterol is claimed to be the precursor for bile acid synthesis in man [42], in our experiments BRL 26314 had no consistent effect on the activity of cholesterol 7α-hydroxylase (Table 5), the enzyme usually believed to be rate-limiting for bile acid production. However, as this enzyme is known to exhibit circadian periodicity and as we studied the activity at the nadir, it is possible that a diurnal variation in response might be present. The increased bile acid secretion is confined to cholic acid, which may imply a specific activation of the 12-hydroxylation step, and is associated with hypertrophy of the biliary ducts in rats but not rabbits which we consider to be work-induced (results to be published separately).

It is not easy to predict the extent to which a change in serum HDL concentration is translated into a change in cholesterol turnover because of the possibility of amplification by increased shuttling within the circulation. Because of this and because, when comparing classes of agents, a rise in HDL can be related, either to cholesterol turnover (BRL 26314), or decreased transport (ANIT), we consider that it is not sufficient merely to measure the static concentration of HDL. More detail on the mode of action of BRL 26314 might be obtained by further fractionation of HDL although 80% of HDL cholesterol is carried by HDL_2 in rats [43].

BRL 26314, as a representative of a series of chemically novel phenylalanine analogues [13], produces an effect on lipoproteins distinct from those produced by previous classes of compounds con-

sidered to be of interest with respect to lipid metabolism. Not only is the mechanism of action dissimilar to the standard 'hypolipidaemic' agents but it is also unlike some other recently described phenylalanine analogues claimed to be inhibitors of β-hydroxy-β-methylglutaryl-CoA reductase [44] and other phenylalanine-related compounds which inhibit mevalonate pyrophosphate decarboxylase and mevalonate phosphate kinase [45].

The profile of activity shown by BRL 26314 in rats is encouraging and if reproduced in patients with hyperlipidaemia would be expected, in due course, to decrease arterial cholesterol, atherosclerosis and thence the risk of coronary heart disease. It is difficult to induce atherosclerosis in rats, so to determine the influence of BRL 26314 on a pathological endpoint, the compound has also been studied in rabbits and these experiments form the basis of the following presentation.

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