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Comparison of the Lipoprotein Profiles and the Effect of *N*-Phenylpropyl-*N*-Benzyloxy Acetamide in Primates (38473)

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The use of primates, as a species comparable to man, in the study of bile acid metabolism (1), cholesterol (2), and the changes related to the risk of coronary heart disease (CHD) (3), has been clearly shown.

Although atherogenic diets cause an increase in serum cholesterol, especially in the low density lipoproteins (LDL) in primates (4) and dietary fats alter the lipoprotein composition (5, 6), it is unclear why different species of primates respond differently to atherogenic diets (7) and whether dietary lipids, especially when in excess, are distributed between the serum lipoproteins in a comparable fashion to that in man.

Several studies of the lipoprotein profiles of monkeys have been reported (5–8) but few comparative studies of the profiles from different species are available (9, 10).

Since *N*-α-phenylpropyl-*N*-benzyloxy-acetamide (W1372) has been reported to decrease serum cholesterol and phospholipids in Squirrel and Cebus monkeys (11) and to decrease the extent of atherosclerotic lesions in Squirrel monkeys fed an atherogenic diet, it was of interest to further investigate the lipoprotein changes in several species of primates treated with W1372.

This investigation reports a comparative study of the lipoprotein profiles obtained by analytical centrifugation and agarose gel electrophoresis of Squirrel, Cebus and Rhesus monkeys, and the effects of W1372 on the lipoprotein profiles.

Changes in the lipoprotein profiles of Cebus monkeys maintained on an atherogenic diet and given W1372 were investigated. Concomitant changes in the liver lipids in untreated and W1372 treated animals are also reported.

Methods. Young monkeys, Saimiri Sciureus, Cebus Apella, and Macaca Mulatta were obtained from the Tarpon Zoo (Tarpon Springs, FL) and were housed individually and held in quarantine for 6 wk before use. The monkeys were maintained on Purina Chow supplemented with butter, applesauce, dextrin and vitamins as reported previously (11). The daily food intake and initial and final body weight were determined. One group of Cebus monkeys was maintained on a hypercholesterolemic diet containing 2.37 g of cholesterol and 9.46 g of butterfat per 100 g of diet (12).

W1372 was given, except where noted, daily by gavage. The dose was increased progressively in Squirrel monkeys from 40 to 120 mg/kg and maintained at 120 mg/kg for 4 wk. Cebus and Rhesus monkeys were given an initial dose of 20 mg/kg and this was then increased progressively 30 mg/kg/wk to a final dose of 330 mg/kg in Cebus and 210 mg/kg in Rhesus monkeys. The final dose was based on previously determined levels of toxicity for the three species (11). Blood samples were taken weekly. Cebus and Rhesus monkeys were injected intravenously with labelled precursors 24 hr prior to sacrifice. At sacrifice, the liver was chilled, extracted and the lipid and radioactive content determined.

The lipoproteins were also separated for analytical ultracentrifugation by a modified procedure of Del Gatto *et al.* (13). Serum

(1–2 ml) was dialysed overnight at 5° versus NaBr solution of d = 1.20 (20°) pH 7.4, 0.02 *M* Tris, 0.02% EDTA. A similar NaBr solution was then layered over the dialysed serum and spun at 40,000 rpm for 20 hr at 18°. The top layer of each tube was collected and the volumes measured (usually 1.0–1.5 ml). The runs were made in a Spinco ultracentrifuge at 50,740 rpm and 20° in double sector cells with NaBr solution in one sector to establish baselines. Flotation rates are expressed in Svedbergs and refer to flotation at 20° in a solvent density of 1.20. Concentrations of the lipoprotein fractions were estimated from area measurements of the enlarged tracings of the schlieren patterns. Refractive index increments of the lipoprotein classes used to convert areas to concentration values, were computed from literature values (14), the refractive index of NaBr solution, and the equation of Cassassa and Eisenberg (15).

Lipoproteins were separated electrophoretically on 0.5% agarose gel on microscope slides in barbitone-acetate buffer 0.1 *M*, pH 8.6 as described previously (16, 17). After staining for lipid, photographing, and scanning in a densitometer, the slides were placed in 5% TCA for 10 min and then washed in 2:1 chloroform–methanol to remove the lipid dye. The same slides were then stained for proteins and photographed and scanned again. This technique permitted direct comparison of the position of lipoprotein bands with respect to albumin in each individual serum.

Lipoproteins were also separated by dextran sulphate fractionation into dextran sulphate insoluble low density lipoproteins (DPL) and the dextran sulphate soluble high density lipoproteins (DSL). This procedure has been reported to separate the low and high density lipoproteins in several species of monkeys (18) precipitating the low density β-lipoproteins ($d \leq 1.06$) (19).

The cholesterol, phospholipid and triglyceride contents were determined in the serum and lipoprotein fractions as described previously (20).

Tissue lipids. Part of the liver was homogenized, extracted twice at 60° with ethanol: ether, the lipid contents isolated, and the radioactivity and lipid contents were determined in the extract as described previously (20). The phospholipids were quantitatively precipitated with acetone saturated with magnesium chloride and their content and radioactivity determined (20).

Results. It has been reported that although the food intake is decreased in Squirrel monkeys receiving W1372, changes produced by W1372 are still evident in pair fed monkeys (11). In this study when the food intake of Squirrel and Rhesus monkeys was reduced by two thirds over a period of three weeks, but the supply of vitamins and salts was unaltered, neither the serum cholesterol (Rhesus, initial 149 ± 6, final, 144.5 ± 6; Squirrel, initial, 182 ± 13, final, 147 ± 9 mg/100 ml) nor the phospholipids (Rhesus, initial, 222 ± 11, final, 232 ± 13; Squirrel, initial 182 ± 13, final, 207 ± 10 mg/100 ml) were altered.

W1372 given by gavage or added to the diet produced a comparable reduction in the DPL cholesterol and phospholipid in Squirrel monkeys (Table I). W1372 treatment lowered the phospholipid in the DPL and DSL of Cebus monkeys, while apparently increasing the DSL phospholipid in Rhesus monkeys (Table I). It should be noted that changes in the lipoprotein fractions separated by dextran sulphate fractionation were consistently found during weekly sampling.

The electrophoretogram and ultracentrifugal patterns obtained from Rhesus and Cebus monkeys are shown in Fig. 1 and 2. The lipoprotein profiles for each monkey are given separately since differences between individual animals were evident.

Chylomicrons were not observed either by agarose gel electrophoresis or in the analytical ultracentrifuge in Cebus, Rhesus and Squirrel monkeys.

The patterns obtained with samples from Squirrel monkeys, both untreated and treated were similar to those shown in Fig. 1 D, H. None of the monkeys except one Squirrel monkey displayed VLDL on the normal diet.

The Squirrel monkeys displayed only one LDL component, while Cebus (Fig. 2) and some Rhesus monkeys (Fig. 1, E and F) displayed two distinct LDL components.

No clear separation of pre-β-lipoprotein

TABLE I. EFFECT OF W 1372 ON THE SERUM LIPIDS IN THE SQUIRREL, RHESUS AND CEBUS MONKEYS.

	Cholesterol		Phospholipid	
	DPL[b]	DSL[b]	DPL[b]	DSL[b]
Squirrel monkey[a]				
Untreated (5)	110 ± 18[c]	88 ± 4	67 ± 5	126 ± 16
W 1372 by gavage (5)	69 ± 10	87 ± 10	46 ± 6*	96 ± 13
W 1372 in diet (5)	62 ± 11*	67 ± 7	43 ± 3**	83 ± 8**
Rhesus monkey[a]				
Untreated (4)	104 ± 10	58 ± 5	58 ± 7	132 ± 12
W 1372 by gavage (3)	87 ± 17	71 ± 0	46 ± 13	187 ± 9*
Cebus monkey[a]				
Untreated (3)	87 ± 8	64 ± 4	64 ± 4	110 ± 6
W 1372 by gavage (3)	71 ± 4	56 ± 4	41 ± 8*	82 ± 8*

* $P \leq 0.05$. ** $P \leq 0.01$.

[a] Squirrel monkeys were given 0.3% w/w in the diet or 120 mg/kg BW by gavage for 4 wk; Rhesus and Cebus monkeys were given daily 330 mg/kg BW and 210 mg/kg BW respectively by gavage.

[b] Dextran sulphate insoluble fraction (DPL); Dextran sulphate soluble fraction (DSL).

[c] Results given as mg lipid per 100 ml serum and expressed as mean and standard error. The number of monkeys per group given in parentheses.

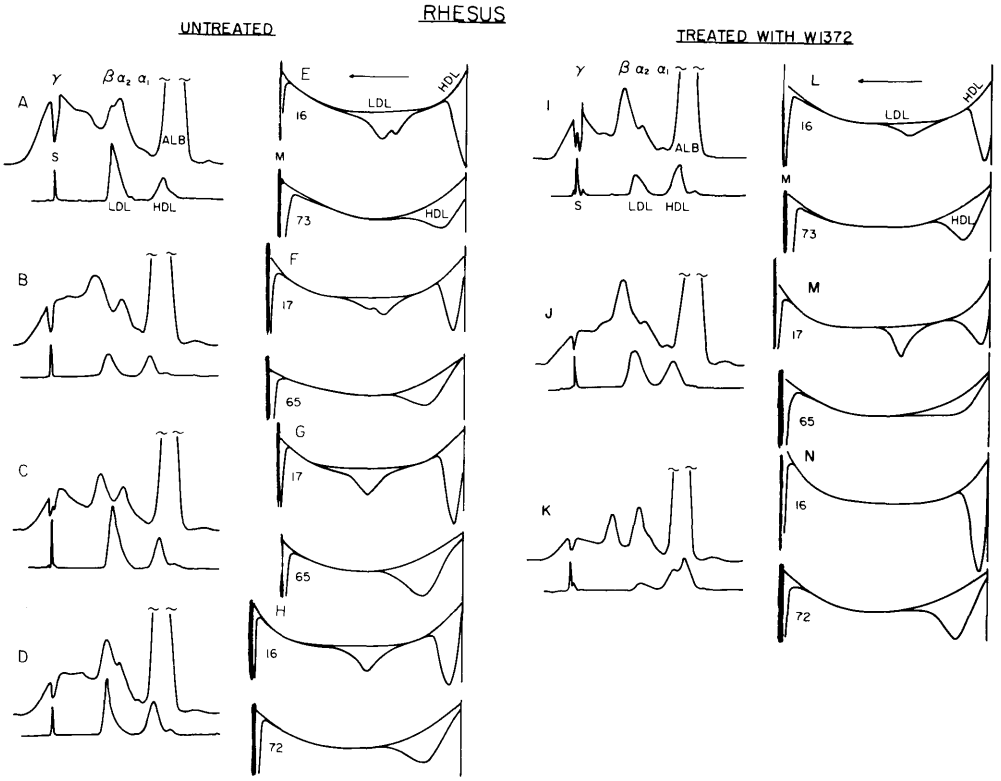

FIG. 1. Agarose gel electrophoretic and analytical ultracentrifuge patterns of the serum lipoproteins of Rhesus monkeys of the same size and age. Electrophoretic and ultracentrifuge patterns from the same animals are side by side (e.g., A is electrophoretic and E is corresponding ultracentrifuge pattern). Arrow indicates flotation direction; M is meniscus position, and time is in minutes; S indicates the electrophoretic sample slot.

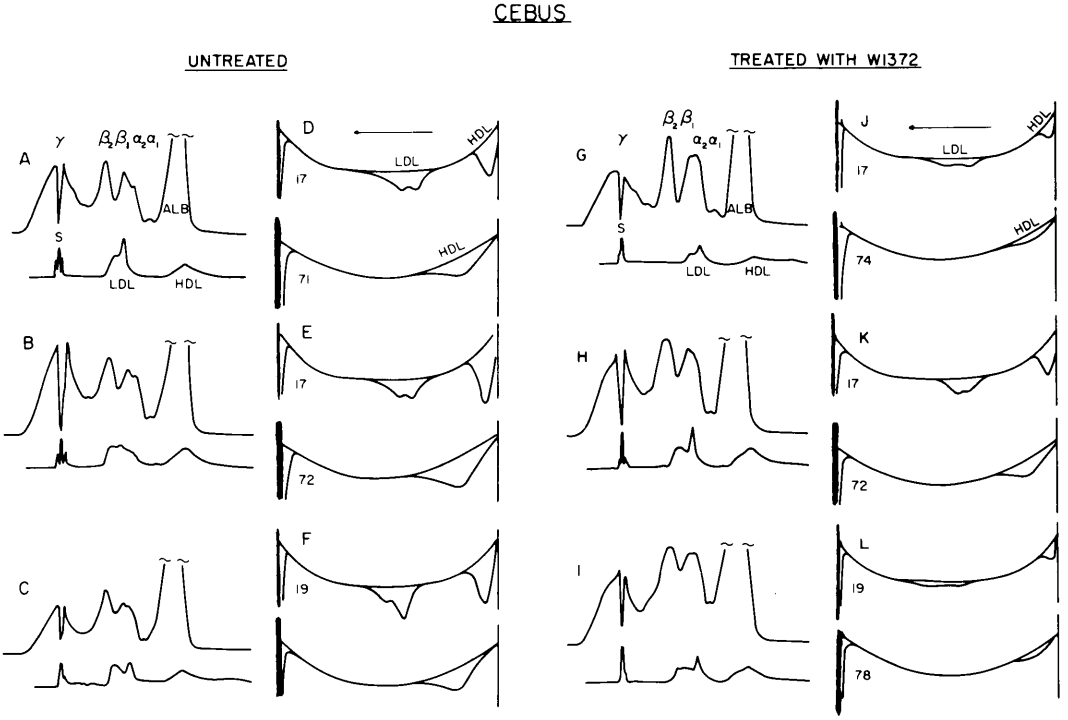

FIG. 2. Agarose gel electrophoretic and analytical ultracentrifuge patterns of the serum lipoproteins of Cebus monkeys of the same size and age. Electrophoretic and ultracentrifuge patterns from the same animals are side by side (e.g. A is electrophoretic and D is corresponding ultracentrifuge pattern. Time is in minutes; S indicates the electrophoretic sample slot.

was found on agarose gel electrophoresis, except in the Squirrel monkey mentioned above. With Cebus monkeys, a considerable amount of lipoprotein was present in the pre-β region but without other supporting data it cannot be equated with VLDL.

The flotation rates given in Table II show that LDL and HDL are quite comparable to values reported for human serum (13). It would appear that the rate for HDL of W1372 treated Cebus is low, even more so if the one animal with a normal rate (2.4 S) is not included.

The flotation rates of LDL and LDL_2 from the control animals corrected to NaCl solvent density of 1.063 and 26° would be about 4.1 S and 1.7 S respectively, assuming the same partial specific volumes found for similar lipoproteins of pig serum (22). These values are in the range of the 0–12 LDL class of human serum. It probably requires a solvent density of 1.20 to clearly resolve the two LDL components as was shown by Janado *et al.* (22) for pig serum lipoproteins.

A decrease in the amount of LDL was found in all the monkeys given W1372 (Table II). The LDL_2 was not found in W1372 treated Rhesus monkeys. In Cebus monkeys, W1372 decreased both LDL_1, LDL_2, and the HDL concentration.

In all three species, W1372 treatment increased the triglyceride and cholesterol content in the liver (Table III). It should be noted, however, that W1372 does not inhibit cholesterol biosynthesis in the liver (23).

As shown in Table IV, W1372 increased the liver weight and the total phospholipid in all three species, but only increased the phospholipid concentration in the liver in Cebus and Squirrel monkeys. A quantitative recovery of the phospholipid was obtained by acetone Mg^{2+} precipitation. The incorporation of labelled choline into the liver phospholipids, was increased by W1372 in the three species, while the specific activity of the phospholipids was reduced only in Cebus monkeys.

Feeding an atherogenic diet to Cebus

TABLE II. EFFECTS OF W1372 ON LIPOPROTEIN CONCENTRATION AND FLOATATION RATES IN SQUIRREL, RHESUS, AND CEBUS MONKEYS.[a]

	Lipoprotein concentration				HDL
	LDL^c	LDL_2	$LDL^c + LDL_2$	HDL	$LDL^c + LDL_2$
Squirrel					
(3) Control	104 ± 32	—	104	194 ± 71	1.86
(4) Treated	68 ± 19	—	68	200 ± 102	2.94
Rhesus					
(4) Control	98 ± 39	60 ± 6	158	198 ± 24	1.25
(3) Treated	64 ± 57	—	64	213 ± 49	3.33
Cebus					
(3) Control	70 ± 29	67 ± 19	137	169 ± 25	1.23
(3) Treated	23 ± 16	29 ± 11	62	48 ± 27	0.77
	Lipoprotein floatation rates[b]				
	LDL_1	LDL_2		HDL	
Squirrel					
(3) Control	20.9 ± 0.8	—		2.9 ± 0.2	
(4) Treated	22.7 ± 2.9	—		2.6 ± 0.2	
Rhesus					
(4) Control	23.0 ± 1.3	18.3 ± 1.1		2.8 ± 0.4	
(3) Treated	23.2 ± 3.0	—		2.5 ± 0.2	
Cebus					
(3) Control	22.3 ± 2.3	19.3 ± 0.6		2.3 ± 0.1	
(3) Treated	24.5 ± 0.4	20.3 ± 0.4		1.3 ± 1.0	

[a] The lipoprotein concentrations (mg/100 ml of serum) were estimated from the schlieren patterns. The number of monkeys per group given in parentheses. The results given by mean ± standard error. LDL_2 of Rhesus control was present in only two animals.

[b] Rates are in Svedbergs measured in NaBr of *d* 1.20 at 20°.

TABLE III. EFFECT OF W1372 ON THE LIPID CONTENT IN THE LIVER OF CEBUS, RHESUS AND SQUIRREL MONKEYS.[a]

	Cholesterol		Triglyceride	
	mg/g	mg/liver	mg/g	mg/liver
Cebus monkeys				
Untreated (3)	3.8 ± 0.4	157 ± 19	10.1 ± 3.2	437 ± 165
Treated (3)	4.7 ± 0.2	359 ± 18*	18.0 ± 1.9	1,385 ± 140*
Rhesus monkeys				
Untreated (4)	1.3 ± 0.8	120 ± 35	28 ± 6	2,060 ± 328
Treated (3)	1.3 ± 0.2	178 ± 39	95 ± 30	9,515 ± 3340
Squirrel monkeys				
Untreated (5)	5.6 ± 0.5	77 ± 8	13.5 ± 2.3	184 ± 28
Treated (5)	6.7 ± 0.9	116 ± 6.5**	15.2 ± 2.6	284 ± 67

* $P \leq 0.05$. ** $P \leq 0.01$.

[a] Monkeys treated as in Table I. Livers were removed and the lipid content determined as described in the text. Number of monkeys per group given in parentheses. Result given as mean and standard error.

monkeys (Table V), apart from increasing the DPL and DSL cholesterol, also markedly increased the DPL phospholipids. The elevated levels of both cholesterol and phospholipids were partly reduced by treatment with W1372.

On analytical ultracentrifugation of the serum of untreated Cebus monkeys fed an

TABLE IV. THE EFFECT OF W1372 ON THE INCORPORATION OF LABELLED CHOLINE INTO LIVER PHOSPHOLIPIDS IN CEBUS, RHESUS AND SQUIRREL MONKEYS.[c]

	Body weight		Liver			
	Final	Wet weight	Radioactivity			Phospholipid[a]
	kg	g	dpm × 10^{-3} (3H)	mg/liver	mg/g	dpm/mg × 10^{-3} (3H)
Cebus[b]						
Untreated (3)	2.04 ± 0.08	41.5 ± 4.0	13 ± 1	49 ± 6	1.22 ± 0.04	273 ± 10
Treated (3)	1.89 ± 0.04	76.8 ± 6.8**	21.6 ± 1.5**	139 ± 4**	1.83 ± 0.07**	158 ± 15**
Rhesus[b]						
Untreated (4)	4.22 ± 0.29	91 ± 12	3.2 ± 0.3	31 ± 5	0.35 ± 0.04	113 ± 17
Treated	3.22 ± 0.37	135 ± 28	8.8 ± 0.4**	60 ± 10*	0.45 ± 0.06	156 ± 28
Squirrel[b]						
Untreated (5)	0.77 ± 0.04	13.6 ± 0.8	1.8 ± 0.2	12 ± 1	0.71 ± 0.04	141 ± 15
Treated (5)	0.66 ± 0.04	18.2 ± 1.9	3.0 ± 0.2**	21 ± 1**	1.12 ± 0.04**	198 ± 33

* $P \leq 0.05$. ** $P \leq 0.01$.

[a] The radioactivity and phospholipid content in the liver was determined in the resuspended extract after acetone Mg^{2+} precipitation as described in the text.

[b] Each monkey was injected intravenously with 50 μCi (Me. ^3H) choline. Squirrel monkeys were killed after 1 hr; Cebus and Rhesus monkeys after 24 hr.

[c] Results given as mean ± standard error. Number of monkeys in pareutheses.

TABLE V. SERUM PHOSPHOLIPID AND CHOLESTEROL CONTENT IN CEBUS MONKEYS FED A STANDARD ATHEROGENIC DIET.[a]

	Cholesterol		Phospholipids	
	DSL	DPL	DSL	DPL
Standard diet (5)	75 ± 11	84 ± 11	140 ± 17	96 ± 6
Standard diet + W1372 (5)	55 ± 5	59 ± 14	89 ± 9*	77 ± 13
Atherogenic diet (5)	160 ± 16	318 ± 77	157 ± 22	256 ± 44
Atherogenic diet + W1372 (5)	56 ± 7**	179 ± 36	85 ± 13*	149 ± 23

[a] Results expressed as mg/100 ml and given as mean ± standard error. Number of monkeys per group given in parentheses.

* $P \leq 0.05$. ** $P \leq 0.01$.

atherogenic diet (Fig. 3), the LDL and HDL peaks had flotation rates of 26 and 2.9 S, respectively, which represented 273 mg of LDL and 362 mg of HDL/100 ml of serum. In a W1372 treated Cebus monkey fed the atherogenic diet, the LDL peaks had flotation rates of 42, 34 and 27 S which contained 240, 246 and 469 mg of LDL, respectively, per 100 ml of serum, while the HDL had 3.2 S and 283 mg. These values clearly exceeded those of Cebus monkeys on normal diets. In both the untreated and treated Cebus monkeys, the HDL and LDL content was abnormal and the LDL of treated Cebus had multiple peaks. Agarose gel electrophoresis of the lipoproteins of Cebus monkeys on the atherogenic diet, both treated and untreated showed abnormal patterns (Fig. 3). Bands labelled one and two probably correspond to LDL and HDL, respectively, but each had excessive mobility.

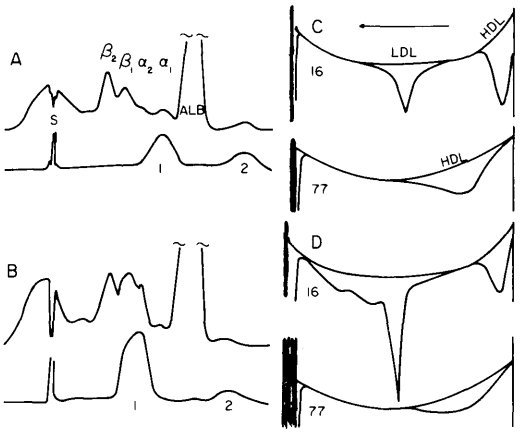

FIG. 3. Agarose gel electrophoretic (left) and analytical ultracentrifuge (right) patterns of the serum lipoproteins of Cebus monkeys of the same size and age. A and C, B and D patterns from corresponding animals. Arrow indicates flotation direction; time is in minutes; S indicates the electrophoretic sample slot. A and C monkeys on a high fat diet; B and D monkeys on a high fat diet with W1372.

Discussion. In this study of the flotation rates and lipoprotein profiles in three species of monkey, the Cebus and Rhesus monkeys had two distinct LDL components: LDL_1, LDL_2; but only one was present in the Squirrel monkey. On the dietary regime in this study, no VLDL was found in any of the three species, which confirms the findings of others (24, 25) that little VLDL is present in non-human primates.

Only one Squirrel monkey exhibited a small amount of VLDL and pre-β-lipoprotein. Pre-β-lipoproteins have been previously reported in Rhesus monkeys which showed a type II lipoproteinemia (8). However, without other supporting data, lipoprotein in the pre-β region found in one Rhesus monkey given W1372 (Fig. 1K) and Cebus monkeys (Fig. 2) cannot be equated with pre-β lipoproteins.

In non-human primates on atherogenic diets the increase in cholesterol may be carried in the LDL fraction (26). A similar increase in the LDL component occurred in Cebus monkeys fed an atherogenic diet in this study. Dietary induced changes in the lipoprotein profiles have been reported in Cebus (5) and Rhesus monkeys (6). Changes in the composition of the DPL (LDL) fraction in the Cebus monkeys fed an atherogenic diet was clearly reflected in agarose gel patterns.

In primates, differences in cholesterol

metabolism between species may be related to differences in their absorption of cholesterol (27), while their response to fat of cholesterol also appears to be different. Recently, Corey *et al.* (7) reported Cebus monkeys were more sensitive to saturated fat, while Squirrel monkeys showed a greater hypercholesterolemic response to dietary cholesterol.

If excess cholesterol is carried by the LDL and not the VLDL in monkeys, the finding of only one LDL component in the Squirrel monkey may partially explain the susceptibility of the Squirrel monkey to atherosclerosis. The absence of a correlation between the serum cholesterol level and the degree of sudanophilia in Cebus monkeys (24) may be partially related to the presence of an LDL_2 fraction which can transport cholesterol yet may less readily infiltrate into the aorta than the LDL_1 fraction.

All monkeys given W1372 showed a decrease in the amount of LDL and a decrease in the HDL in Cebus monkeys. The LDL_2 fraction was not found in W1372 treated Rhesus monkeys, while both the LDL_1 and LDL_2 fractions were reduced in Cebus monkeys.

Accumulation of cholesterol and triglyceride, together with phospholipids in the liver of Squirrel monkeys, and also decreased synthesis of liver phospholipids in Cebus monkeys, indicated W1372 prevented the release and/or synthesis of the lipoproteins from the liver.

As the Cebus monkey is more sensitive to saturated fat than the Squirrel monkey (7) and shows distinct changes in lipoprotein profile with diet, the multiple classes of lipoproteins in W1372 treated Cebus monkeys maintained on an atherogenic diet suggests W1372 could be used to investigate the relative importance of different lipoproteins in the development of atherosclerosis in Cebus monkeys.

Summary. Analytical ultracentrifugation showed Cebus and Rhesus monkeys had two low density components while only one was present in Squirrel monkeys. In untreated or W1372 treated monkeys, neither chylomicrons nor very low density lipoproteins were detected on analytical ultracentrifugation. Chylomicrons were not observed on agarose gel electrophoresis.

Ultracentrifugal analysis showed W1372 treatment decreased the amount of LDL in all animals and also the HDL in Cebus monkeys on an atherogenic diet. Both untreated and W1372 treated Cebus monkeys on an atherogenic diet had abnormal amounts of LDL and HDL, while the LDL in treated animals occurred as multiple peaks. This was also evident on agarose gel electrophoresis. Accumulation of lipids in the liver and decrease of serum lipids indicated W1372 prevented release of lipoproteins from the liver.

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