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THE MECHANISMS UNDERLYING THE HYPOLIPIDAEMIC EFFECTS OF ATROMID S, NICOTINIC ACID AND BENZMALECENE—I

THE METABOLISM OF FREE FATTY ACID–ALBUMIN COMPLEX BY THE ISOLATED PERFUSED LIVER

M. A. MISHKEL* and W. F. WEBB

School of Medicine, The University of New South Wales

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Abstract—The metabolism of 1-^{14}C palmitic acid complexed with albumin was studied utilizing the isolated perfused rat's liver obtained from male rats fed a diet containing a hypocholesterolaemic drug. The three drugs examined were the ethyl ester of chlorophenoxyisobutyrate (C.P.I.B.), nicotinic acid and benzmalecene which were added to a standard rat diet at a concentration of 0·2, 1 and 0·2% w/w, respectively. There were no differences in the hepatic uptake of the palmitic acid by the normal and experimental groups. The livers from rats fed C.P.I.B. were heavier than normal, but the increase in weight was not due to any great increase in hepatic lipids. A fall in perfusate triglyceride level occurred over the 3 hr perfusion period in all groups, but was greatest in the C.P.I.B. fed group. This finding coupled with the greater incorporation of radioactivity into the hepatic triglyceride fraction together with the normal levels of perfusate triglyceride activity,was interpreted as showing a decreased hepatic retransport of triglyceride by the livers of the C.P.I.B. fed group. Both the nicotinic acid and benzmalecene groups showed an increased oxidation of the palmitic acid, which might account in part for the hypolipaemic effect of these two drugs.

SINCE the introduction of nicotinic acid by Altschul *et al.*[1] for use in lowering serum cholesterol levels in man there has been considerable interest in finding the ideal hypocholesterolaemic drug. The ultimate aim has been to produce regression of the atheromatous plaque and to influence favourably the clinical course of the atherosclerotic vascular disease. The former has been achieved by means of dietary manipulation and drugs in the experimental animal[2–4] but to date there has been no conclusive evidence of the latter in man.

Even with a well-studied drug like nicotinic acid there is little unanimity concerning its mode of action in lowering serum cholesterol levels. The hypolipidaemic effect of any drug will depend upon one or more of the following mechanisms: a decreased absorption of lipids; a decreased hepatic synthesis of lipids; an increased hepatic oxidation or an increased degradation and excretion of lipids in the bile; a decreased hepatic lipid secretion or decreased mobilization from peripheral, especially adipose, tissues. Another less documented mechanism is that drugs may cause a redistribution of serum lipids into other tissues.

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The present work has been designed to study the possible modes of action of three drugs which have been used to lower serum cholesterol levels in man, viz. atromid S, nicotinic acid and benzmalecene. In this paper we have studied by means of the isolated perfused rat's liver technique, the effect of these three drugs on the metabolism of 1-^{14}C labelled palmitate complexed with albumin.

MATERIALS AND METHODS

Drugs

The drugs used in all experiments were:

(i) nicotinic acid (B.D.H.)—laboratory reagent grade;
(ii) atromid S—ethyl ester of *p*-chlorophenoxyisobutyrate, C.P.I.B. (I.C.I.)—pharmaceutical grade;
(iii) benzmalecene—*n*- [1-methyl-2,3—bis (*p*-chlorophenyl)—propyl] maleamic acid (M.S.D.)—pharmaceutical grade.

Diets

The liver donor rats in the control group were fed ground rat nuts (composition: carbohydrate 60%, fibre 6%, fat 4%, protein 15%, moisture 10%, salts and vitamins 5%). The experimental groups were fed ground rat nuts incorporating either 1% nicotinic acid, 0·2% C.P.I.B. or 0·2% benzmalecene (all w/w) for 10 days.

Animals

All animals used as liver donors and as bleeders for provision of the blood for the perfusions were male albino rats bred in the Animal Breeding Establishment of the University of New South Wales. They were fed right up till the time of the experiments. The surgical technique, perfusion apparatus and perfusion fluid were as outlined by Mishkel and Morris.[5]

Biochemical methods

The perfusate plasma lipids were estimated as follows: cholesterol by the method of Brown,[6] triglyceride by the method of Carlson and Wadstörm,[7] and phospholipid by the method of Zilversmit and Davis.[8] The extraction of liver lipids was performed immediately at the end of the perfusion, an aliquot of the liver being ground and extracted with 20 vol. of 2 : 1 chloroform : methanol (v/v) for 24 hr in an atmosphere of nitrogen. After filtration, the extract was treated as described by Olivecrona.[9]

The total lipids in the final chloroform extract were measured gravimetrically and the constituent lipids were estimated by the methods used for serum lipids. Thin-layer chromatography of the serum and hepatic lipids[10] was carried out on 20 × 20 cm plates coated with 250 μ layers of silica gel G (Merck) activated at 120° for 1 hr. The solvent system used was petroleum ether (b.p. 60–80°C):diethyl ether-acetic acid 85:15:1, to a height of 10 cm. Pure compounds and a known mixture (Hormel Institute TLC No. 3 mixture) were run on every plate which was developed with iodine vapour. For confirmation, certain tracks on each plate were sprayed with 10% phosphomolybdic acid and heated to 150° for 30 min. Reproducible separation was obtained for the following substances: phospholipid, cholesterol (plus mono- and diglyceride), free fatty acids, triglyceride and cholesterol esters. The lipid spots were scraped directly into counting vials and 10 ml of scintillant were added (4 g PPO

and 100 mg dimethyl-POPOP in 1 L-toluene). In the case of phospholipids only, the silicic acid was suspended in 4% Cab-O-Sil (Packard) in the above scintillant. The $^{14}CO_2$ which had been absorbed by a solution of N NaOH during the perfusion was counted by the method described by Harlan.[11] All liquid scintillation counting was by means of a conventional Packard liquid scintillation counter. The total perfusate activity was determined by precipitating the perfusate proteins with 10% trichloracetic acid on metal planchets, drying them at 60° and then counting by means of a thin end-window G.M. tube.

The total CO_2 produced during the perfusion was determined by precipitation with barium chloride, washing the precipitate three times with CO_2-free water with intervening centrifugation. The precipitate was dissolved in an excess of 0·1 N HCl and back titrated with 0·1 N NaOH. The substrate for all perfusions was 5 μc 1-^{14}C palmitic acid, specific activity 36·6 mc/m-mole (Radiochemical Centre, Amersham) which was converted to the sodium salt with a small excess of sodium hydroxide, to which was rapidly added freshly obtained rat's serum.

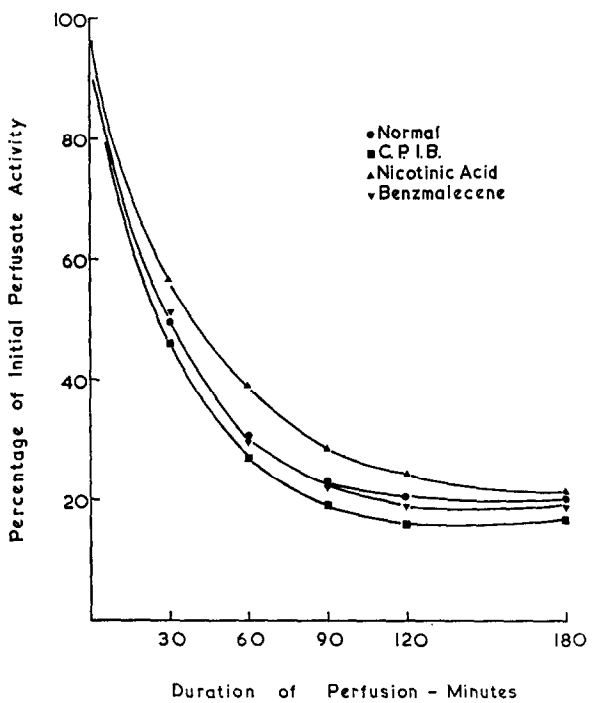

FIG. 1. The perfusate total activity removal curves in normal, C.P.I.B., nicotinic acid and benzmalecene group livers perfused with 1-^{14}C palmitic acid–albumin complex.

RESULTS

Perfusate total lipid activity removal curves

The total lipid radioactivity in the perfusate plasma was determined prior to commencement of the perfusion and at intervals throughout the 3-hr perfusion. Figure 1 shows the mean activity at the various time intervals for the 4 groups of

experiments—normal, nicotinic acid, C.P.I.B. and benzmalecene groups. The fall in percentage activity is very similar in all 4 groups, except that the levels of activity in the nicotinic acid group tended to be higher. The mean extraction coefficient at 30 min (i.e. the percentage activity removed by one passage through the liver) was determined by measuring the activity in simultaneous portal and hepatic vein perfusate samples. The results for normal, C.P.I.B. and benzmalecene groups were found to be 44·0, 48·2 and 45·2 per cent respectively, whereas the extraction coefficient in the nicotinic acid experiments was lower, being 39·3 per cent. These findings could be interpreted as showing that the livers in the normal, C.P.I.B. and benzmalecene groups had comparable rates of uptake of palmitic acid albumin complex (free fatty acid or FFA) whereas the livers in the nicotinic acid group showed some impairment of uptake. If the decreased weight of the livers in the nicotinic acid group is taken into consideration (Table 3) then the uptake of activity by these livers is found to be comparable to the other groups.

Changes in the perfusate lipid concentrations

The concentrations of cholesterol, phospholipid and triglyceride in the perfusate prior to perfusion (P_0) and after 180 min perfusion (P_{180}) are compared in Table 1, for the 4 groups of experiments. There was no significant change in the cholesterol or phospholipid concentration for any experimental group. The triglyceride concentration fell in all experimental groups, and the triglyceride concentration in the P_{180} sample was significantly lower ($P < 0{\cdot}02$) in the C.P.I.B. group than in the normal group. (Table 1).

TABLE 1. CHANGES IN THE MEAN PERFUSATE LIPID CONCENTRATION AFTER 3 HR PERFUSION

Experimental group	Perfusate cholesterol ($P_{180} \times 100/P_0$)	Perfusate triglyceride ($P_{180} \times 100/P_0$)	Perfusate phospho-lipid ($P_{180} \times 100/P_0$)
Normal	104·3 ± 12·3 (17)	78·1 ± 22·0* (17)	106·2 ± 24·1 (7)
C.P.I.B.	100·4 ± 10·0 (9)	55·0 ± 15·0* (7)	99·2 ± 14·4 (3)
Nicotinic acid	100·7 ± 15·8 (12)	80·9 ± 21·7 (14)	98·3 ± 20·6 (13)
Benzmalecene	111·7 ± 13·2 (7)	85·1 ± 21·9 (7)	100·1 ± 24·8 (6)

The mean 180 min (P_{180}) lipid concentrations are expressed as a percentage (± S.D.) of the mean pre-perfusion (P_0) levels. The number of experiments in each group is shown in parentheses.
*$0{\cdot}02 > P > 0{\cdot}01$.

Total CO_2 and $^{14}CO_2$ production

The total 3 hr CO_2 production as well as the total $^{14}CO_2$ production for the 4 experimental groups are shown in Table 2. The total CO_2 production was similar in all 4 groups, but there was an increase in oxidation of FFA in the nicotinic acid and benzmalecene groups and a decrease in the C.P.I.B. group as shown by the total $^{14}CO_2$ activities (Table 2). However, none of these were significant at the 0·05 level.

The effect of the drugs on liver weight and lipids

The addition of 0·2% C.P.I.B. and 1% nicotinic acid to the diet for 10 days led to a failure of the rats to gain weight, and this was particularly marked with rats fed nicotinic acid where considerable weight loss occurred. In the case of nicotinic acid

TABLE 2. THE MEAN TOTAL CARBON DIOXIDE (CO_2) AND $^{14}CO_2$ PRODUCTION DURING THE 3 HR PERFUSION

Experimental group	Total CO_2 (m-moles)	Total $^{14}CO_2$ (counts/min)
Normal (12)	3·96 ± 0·24	506,000 ± 127,000
C.P.I.B. (6)	4·07 ± 0·09	388,000 ± 89,000
Nicotinic acid (11)	3·70 ± 0·07	622,000 ± 92,000
Benzmalecene (7)	4·15 ± 0·09	583,000 ± 227,000

The number of experiments in each group is shown in parentheses.

fed rats, the liver was smaller than normal but the liver:bodyweight ratio was maintained. The nicotinic acid fed rats' livers contained less lipid than the other livers, the decrease being most marked in the triglyceride fraction. The livers from C.P.I.B. fed rats were larger than normal and the liver:body weight ratio was significantly greater than normal ($P < 0·01$) (Table 3). This increase in weight was not due to an increase in lipid as evidenced by the amount of lipid/g of liver, although the separate lipid fractions suggested a small increase in triglyceride. The rats fed 0·2% benzmalecene in the diet gained weight normally, had a normal liver size and total liver lipids.

TABLE 3. THE MEAN BODY WEIGHT, LIVER WEIGHT AND LIPID COMPOSITION OF THE LIVER AFTER 3 HR PERFUSION

Experimental group	Body wt. (g)	Liver wt. (g)	Liver as % of Body wt.	Total liver lipid (mg)	Liver lipid (mg/g liver)	Cholesterol Total mg	Cholesterol per g liver	Triglyceride Total mg	Triglyceride per g liver
Normal (14)	223·6 ±25·4	7·73 ±1·6	3·44 ±0·05	354·0 ±22·1	47·0 ±10·2	14·1 ±2·3	2·08 ±0·5	48·9 ±12·1	7·26 ±1·6
C.P.I.B. (7)	199·0 ±18·9	8·60 ±1·2	4·36 ±0·05	388·7 ±121·0	44·8 ±11·9	15·5 ±2·8	1·78 ±0·8	68·3 ±6·0	8·38 ±2·2
Nicotinic Acid (9)	170·6 ±24·1	5·91 ±1·2	3·45 ±0·04	251·2 ±86·9	42·3 ±10·9	11·2 ±2·6	1·89 ±0·5	28·2 ±10·9	4·29 ±1·4
Benzmalecene (6)	218·0 ±28·0	7·74 ±0·9	3·56 ±0·09	361·3 ±49·7	46·7 ±2·2	13·8 ±1·9	1·79 ±0·1	43·1 ±9·4	5·69 ±1·7

The number of experiments in each group is shown in parentheses.

The incorporation of ^{14}C labelled palmitate into liver lipids

There was no significant difference between the total liver lipid activity in the 4 experimental groups. In all 4 groups the incorporation of ^{14}C activity was greatest into the phospholipid fraction which accounted for some 60–70 per cent of the

activity, the triglyceride fraction making up a further 20–30 per cent. All the other lipid fractions accounted for approximately 5 per cent of the activity (Table 4). The incorporation of ^{14}C activity into the triglyceride fraction was greatest in the livers of C.P.I.B. fed animals ($P < 0.02$, compared with controls).

TABLE 4. THE INCORPORATION OF ^{14}C LABELLED PALMITATE INTO MEAN TOTAL LIVER LIPID ACTIVITY AND THE PERCENTAGE INCORPORATION INTO LIVER LIPID FRACTION ACTIVITY AT THE END OF 3 HR PERFUSION

Experimental group	Total liver lipid activity counts/min	% Incorporation into liver lipids				
		Phospho-lipid	Cholesterol* fraction	Free fatty acids	Triglyceride	Cholesterol ester
Normal (6)	3.66×10^6	69·4	2·8	1·9	24·5†	1·4
	$\pm 0.38 \times 10^6$	±5·9	±0·7	±0·5	±6·5	±0·5
C.P.I.B. (6)	3.80×10^6	62·0	2·3	1·4	33·8†	0·5
	$\pm 0.82 \times 10^6$	±3·3	±0·3	±0·5	±3·3	±0·4
Nicotinic acid (5)	3.75×10^6	65·6	2·9	2·3	27·9	1·3
	$\pm 0.80 \times 10^6$	±2·2	±0·1	±0·6	±2·0	±0·4
Benzmalecene (6)	3.04×10^6	72·3	2·8	3·4	20·5	1·0
	$\pm 0.64 \times 10^6$	±4·3	±1·0	±1·3	±5·2	±0·9

* Cholesterol fraction includes cholesterol, mono- and di-glycerides, the most activity being in the latter two.

† $0.02 > P > 0.01$.

The number of experiments in each group is shown in parentheses.

Retransport of ^{14}C lipid activity from the liver

The majority of the ^{14}C lipid activity of the perfusate plasma at the end of the 3-hr perfusion was found in the free fatty acid fraction which accounted for 40–50 per cent of the activity. There had been considerable retransport of labelled lipid as phospholipid and triglyceride which accounted for approximately 30 and 20 per cent of the lipid activity, respectively (Table 5). There was however, no significant difference in the pattern of retransport of the lipid activity.

TABLE 5. THE MEAN PERCENTAGE DISTRIBUTION OF LIPID ACTIVITY IN THE PERFUSATE AFTER 3 HR PERFUSION

Experimental group	Activity remaining (counts/min)	The % activity present as				
		Phospho-lipid	Cholesterol fraction*	Free fatty acid	Triglyceride	Cholesterol ester
Normal (5)	1.44×10^6	33·1	8·2	40·7	18·0	—
	$\pm 0.30 \times 10^6$	±3·8	±1·3	±7·2	±6·4	—
C.P.I.B. (5)	1.22×10^6	28·3	5·9	48·6	17·2	—
	$\pm 0.30 \times 10^6$	±6·6	±2·0	±12·6	±13·5	—
Nicotinic acid (6)	1.52×10^6	27·8	6·1	44·3	21·8	—
	$\pm 0.23 \times 10^6$	±9·5	±1·9	±11·6	±11·6	—
Benzmalecene (6)	1.37×10^6	27·4	7·7	47·3	17·6	—
	$\pm 0.26 \times 10^6$	±7·0	±3·3	±15·0	±12·3	—

* Cholesterol fraction includes cholesterol, mono- and di-glycerides, the most activity being in the latter two.

The number of experiments in each group is shown in parentheses.

DISCUSSION

Nicotinic acid, the most studied of the three hypolipaemic drugs used in the present experiments, has been in clinical use as a hypocholesterolaemic agent for just over a decade. In minute amounts it is an essential component of two coenzymes, diphosphopyridine nucleotide and triphosphopyridine nucleotide which are involved in carbohydrate, lipid and protein metabolism. The hypocholesterolaemic doses used in man are usually of the order of 3–6 g/day and these doses are one-fifth to one-tenth of that fed to the rats in the present experiments on a weight for weight basis.

The use of the rat to study the hypolipidaemic effect of nicotinic acid has been criticized[12] because of the drugs' relative lack of effect on serum cholesterol in this species.[13] However, a considerable amount of information has been gained on the effect of nicotinic acid on lipid metabolism in the rat. Friedman and Byers[14] suggested that the drug's chief efficacy resided in its anorectic properties. This has been denied by Hoffer[15] but a loss in both body and liver weight whilst on this drug was a feature of the present experiments. Kritchevsky *et al.*[13] studied the effect of both nicotinic acid fed to rats and added *in vitro* on the oxidation of cholesterol, pyruvate and octanoate by rat liver mitochondria. Their results concerning the oxidation of pyruvate and octanoate are relevant to our results obtained with palmitate. They found that nicotinic acid had little effect on the oxidation of sodium pyruvate-2-^{14}C and had a slight inhibitory effect upon the oxidation of sodium octanoate-1-^{14}C. Perry[16] using rat liver slices to which had been added nicotinic acid *in vitro*, showed that under the influence of this drug liver slices converted more 2-^{14}C acetate into $^{14}CO_2$ than did control slices. In addition Perry[16] showed that nicotinic acid depressed the incorporation of acetate into cholesterol and fatty acids and he interpreted his results as showing that the drug favoured lipid oxidative rather than synthetic pathways. The present experiments using palmitate also showed a favouring of oxidative pathways when nicotinic acid was added to the diet, since the highest CO_2 specific activities were found in the nicotinic acid fed group. All liver lipids were decreased in the nicotinic acid fed group, but when a correction was made for the reduced liver mass, the triglyceride fraction was the only one decreased. However, there was no reduction in the total liver lipid activity, no alteration in the pattern of incorporation of ^{14}C activity into any of the liver lipids, nor the subsequent retransport of the labelled plasma lipids. The present experiments were not designed to confirm the reduction of free fatty acid mobilisation by nicotinic acid,[17] but they do show that there is no impairment of FFA uptake by the isolated liver of rats fed this drug.

Benzmalecene, N—[1-methyl-2,3-bis (*p*-chlorophenyl)—propyl] maleamic acid, has been used experimentally in the treatment of hypercholesterolaemia and gout. It has been shown that benzmalecene given orally is capable of reducing the serum cholesterol in the rat[18] in the dog[19] and in man.[20, 21] Huff and Gilfillan[18] showed that it was a potent inhibitor of the incorporation of 2-^{14}C-mevalonic acid into cholesterol by rat liver homogenates. Sachs *et al.*[21] observed that the serum neutral fat levels rose during clinical trials in hypercholesterolaemic and normocholesterolaemic patients and they believed that this was due to a diversion of available acetate or acetoacetate from cholesterol to triglyceride synthesis. Unfortunately in both clinical studies, gastrointestinal side effects precluded the long term administration of benzmalecene.

In the present experiments benzmalecene did not produce a fall in perfusate cholesterol levels over a period of 3 hr. Benzmalecene did however stimulate oxidation of ^{14}C palmitate as shown by the CO_2 specific activity being higher than that of the normal control group. The rats gained weight whilst on the diet containing 0·2% benzmalecene and there was no change in the liver weight or total liver lipids. Although the total liver lipid activity was less than in the other 3 experimental groups, there were no differences in the incorporation of ^{14}C activity into the various liver lipid fractions. Thus our results do not provide evidence for the hypothesis of Sachs *et al.*[21] that triglyceride synthesis is accentuated at the expense of cholesterol synthesis.

Ethyl chlorophenoxyisobutyrate (clofibrate, atromid S, C.P.I.B.) was originally introduced as an oral preparation in combination with a small amount of androsterone and the combination was found to be successful in lowering serum lipids.[22, 23] Later studies[24, 25] have shown that the active agent of the combination is the C.P.I.B. fraction and the latter is now marketed as atromid S. A considerable body of information concerning this agent is now available especially concerning its relative lack of long term toxicity and its effectiveness in lowering serum lipids in man, particularly in those patients with raised serum triglyceride and low density β-lipoprotein levels.[26] Much less however is known about the mode of action of this hypolipaemic agent. Thorp and Waring[22] quote a personal communication from G. S. Boyd which indicates that C.P.I.B. may inhibit acetate incorporation into cholesterol by rat liver slices *in vitro*. Hellman *et al.*[25] were unable to demonstrate the presence of desmosterol or related sterols in the serum of patients treated with C.P.I.B. so this would appear to rule out a block of cholesterol synthesis at this late stage. Azarnoff *et al.*[27] found that when fed in the diet, C.P.I.B. produced a significant decrease in the incorporation of mevalonic acid 2-^{14}C into cholesterol by rat liver homogenates.

The present experiments have confirmed the enlargement of the liver seen in rats fed C.P.I.B. in the diet[27, 28] and that the hepatic enlargement is not due to an accumulation of large amounts of lipid.[27] Azarnoff *et al.*,[27] found that the increased liver weight is due in part to an increase in protein and phospholipid content in female rats but only protein content in male rats. Unlike these authors no decrease in liver cholesterol or triglyceride was found in the present experiments. The oxidation of 1-^{14}C palmitic acid by the C.P.I.B. fed group livers was less than that found in the control group livers in the present perfusion experiments. Similarly, Azarnoff *et al.*,[27] noted a decreased oxidation of mevalonic acid-1-^{14}C by rat liver homogenates from rats fed C.P.I.B. Although the perfusate triglyceride level fell in all experimental groups, the lowest levels were found in the C.P.I.B. group. This low perfusate level of triglyceride was associated with the greatest hepatic incorporation of ^{14}C palmitate into ^{14}C labelled triglyceride but a normal level of ^{14}C activity in the perfusate triglyceride fraction. These results could be interpreted as a partial failure of hepatic secretion of triglyceride into the perfusate being a cause for the hypolipaemic action of C.P.I.B. Azarnoff *et al.*[27] came to the same conclusion from their results with the isolated perfused liver from C.P.I.B. fed rats.

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