The Effect of the Hypocholesterolemic Drug Clofibrate on Liver Mitochondrial Biogenesis

A ROLE FOR NEUTRAL MITOCHONDRIAL PROTEASES*

ADRIAN R. L. GEAR,† ARLENE D. ALBERT, AND JANA M. BEDNAREK
From the Department of Biochemistry, The University of Virginia, Charlottesville, Virginia 22901

SUMMARY

The hypocholesterolemic drug clofibrate raises hepatic levels of mitochondria by over 100%, with maximum increase at 2 to 4 days after drug administration, and a slow rise continuing at 10 days. This time response was similar to that seen after partial hepatectomy. However, several clear differences exist. During the doubling in mitochondrial content, no change in particle size or specific content of DNA or RNA was observed, whereas during liver regeneration the former decreases by 50% and the latter increases by 300%. The half-life of 5.8 days for normal mitochondria was not altered significantly by clofibrate, using [guanidino-14C]-arginine. However, the initial rate of short term incorporation of [35S]methionine into control mitochondria in vivo was 60% of that for drug-tested animals, while the in vitro efficiency of [14C]leucine incorporation was reversed; mitochondria from drug-treated animals were about 61% as active as those from controls. Enzyme activity of drug-treated animals was not significantly altered for inner membrane or matrix enzymes. However, a 30 to 40% fall in specific activity of three outer membrane enzymes was noted from 2 to 15 days. This resembles changes seen during early liver regeneration.

One striking action of clofibrate was to inhibit the neutral protease activity of mitochondria. Even at 2 days on clofibrate, neutral protease activity was inhibited to 62% of the control value of 0.334 μmole of amino acid per hour per mg of protein. Inhibition was a maximum of 50% at 6 days, and was still at 63% at 20 days. Clofibrate added in vitro had negligible influence on neutral protease activity. The inhibition was independent of lysosomes since the protease activity of purified lysosomes was neither inhibited, nor was acid phosphatase activity in whole homogenates or the purified mitochondrial fractions changed during drug treatment. The endogenous amino acid content of 0.032 μmole per mg protein remained constant during the stimulation in biogenesis.

The results of this study on clofibrate support a hypothesis that net levels of hepatic mitochondria may be controlled by the activity of their own neutral proteases. A possible action is to degrade newly synthesized mitochondrial proteins, which may be structural or binding, such that a lowered protease activity would raise their levels and thereby increase mitochondrial content.

Since the introduction of the hypocholesterolemic drug clofibrate in 1962 (1, 2), an enormous volume of literature has described its useful clinical ability to lower serum levels of both cholesterol and triglycerides. Various hypotheses have been proposed for rationalizing this role. However, none are completely satisfactory. For example, explanations of the mode of action of clofibrate range from decreased absorption of cholesterol and enhanced excretion (3, 4), through accelerated conversion of cholesterol to bile acids, inhibition of cholesterol synthesis (5), and a redistribution of cholesterol between plasma and tissue compartments (6, 7).

An early observation that hepatomegaly occurred during clofibrate administration (8) stimulated interest in liver function, particularly since this organ is so vitally concerned with lipid and cholesterol transport, as well as cholesterol oxidation to bile acids. Subsequent to this, a useful study was reported by Hess et al. (9), on some specific enzyme changes. They noted a dramatic 6-fold increase in hepatic levels of glycerol phosphate dehydrogenase specific activity, a significant (74%) rise in cytochrome c oxidase, as well as a smaller 27% increase in catalase. Strangely, the latter microbody enzyme did not parallel urate oxidase, which fell by 66%. These observations correlated with an early electron microscopy study by Paget (10) who showed an elevated number of normal-sized, but denser mitochondria. More lysosomes were thought to exist, but this could have resulted from staining problems, since the later electron micrographs of Hess et al. (9) strongly supported an elevated number of microbody profiles, not lysosomes. Enzyme estimations (7) supported these latter conclusions. Interestingly, the lack of crystallloid in the microbodies correlated with the depressed urate oxidase activity seen after clofibrate treatment.

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In 1970 Kurup et al. (11) reported a detailed study on the specific ability of clofibrate to raise hepatic levels of mitochondria. They demonstrated that most of the increase in liver weight could be accounted for by mitochondrial protein alone, and that apart from the dramatic stimulation of glycerol phosphate dehydrogenase activity (9), mitochondrial "quality" was not altered by the drug. A subsequent study with the related compound clofenapate (12) also demonstrated clearly that only hepatic levels of mitochondria were increased, not those of the nuclear, microsomal, or supernatant fractions.

This striking ability of clofibrate to raise levels of hepatic mitochondria provides a basis for a general hypothesis of the hypocholesterolemic and hyperlipidemic properties of the drug. It is known that although cholesterol conversion to bile acids occurs primarily in the cytoplasm, the final side chain cleavage to propionyl-CoA and chenoyl-CoA is a mitochondrial step (13, 14). Thus Kritchevsky et al. (15) described how clofibrate dramatically increased the activity of rat liver oxidase (129Ca)cholesterol to 12CO2. However, when their data were normalized per mg of mitochondrial nitrogen, there was no difference between mitochondrial fractions derived from control or clofibrate-treated rats. Consequently, the explanation for these observations probably lies in the ability of the drug to cause a net increase in the amount of mitochondrial protein per animal.

The above experiments might help explain the hypocholesterolemic action of clofibrate by increase in mitochondrial cholesterol oxidation to bile acids and salts, and has been confirmed by others (16), but remains controversial (3). The former study in dogs was by analysis of bile obtained from a Thomas cannula, whereas the latter (3) involved analysis of fecal steroids and bile salts in humans. Another rationalization, still related to increased levels of hepatic mitochondria, could be reduced cholesterol synthesis. Several observations support this idea (5, 17) and specifically pointed to cholesterol synthesis from acetate and not mevalonate as being inhibited, but these findings have been questioned (for a discussion, see Ref 5). Since the biosynthetic steps occur extramitochondrially (15), it could be asked how changes in the amount of mitochondria might influence cholesterol synthesis. Recently, however, Burch and Curran (18) found that the activity of mitochondrial acetocarboxyl-CoA decarboxylase was markedly increased in rats fed a clofibrate diet. The increase was about 65% on a total liver basis, and was quantitatively significant in terms of the net cholesterol synthesis by liver. The authors pointed out that the consequence of the deacylase increase would be to reduce levels of cytoplasmic acetacarboxyl-CoA necessary for mevalonate formation, and would fit with the earlier observations of Gould et al. (5). They also presented evidence for a strong relationship between a reduction in digitonin-precipitable sterols and increased deacylase activity. The result could not be demonstrated in vitro, and thus depended on either a metabolite of clofibrate or some time-dependent mitochondrial function. A recent report, however, describes separate pathways for ketone body formation and cholesterol synthesis (18).

A final involvement for the hypothesis of altered hepatic levels of mitochondria relates to the known hypotriglyceremic action of clofibrate. One of the earliest changes in enzyme activity was that of mitochondrial glycerol 3-phosphate dehydrogenase, which rose 6-fold during drug administration (7, 9). A consequence of this rise could be to reduce cytoplasmic levels of glycerol 3-phosphate needed for triglyceride and phospholipid synthesis. Indeed, Fallon et al. (20) reported a 46% lowering of hepatic levels for rats 2 weeks on clofibrate treatment, although these workers thought the best hypolipemic explanation lay in an inhibition of the acyltransferase enzyme, and not with simple lack of necessary substrate.

This brief survey provides good evidence that the hypolipemic activity of clofibrate could well follow from a net increase in hepatic mitochondria. The effect of this could be either to accelerate cholesterol breakdown to bile acids, or inhibit its synthesis, or both, and to decrease glycerol 3-phosphate availability for triglyceride and phospholipid synthesis needed for very low density lipoprotein formation.

The research to be reported here was undertaken to investigate how clofibrate might act in altering levels of liver mitochondria, and to provide a firm basis for the hypothesis that the hypolipemic action of clofibrate is correlated with an increased liver content of mitochondria. The results of this study support the conclusion that clofibrate inhibits mitochondrial neutral protease activity, and thereby raises levels of newly synthesised protein for incorporation into mitochondria.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

Cytochrome c (type 111), DL-lysergic acid, benzylamine, oxaloacetic acid, kynurenine sulfate, rotenone, p-nitrophenyl phosphate and Tris were all obtained from the Sigma Chemical Co., St. Louis, Mo. NADH, NADPH, and ADP were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Glycylglycine; RNA, purified from Torula, N1NA (from salmon sperm), and N-trila(hydroxymethyl)methyl-2-aminooxalosulfonic acid, were obtained from Calbiochem, Los Angeles, Calif. Lubrol-WX, a non-ionic detergent came from IC Organics Inc., Provincetown, R. I. L-Chantastic acid came from Mann Research Laboratories, New York. L-Ascorbic acid, reagent grade; orcinol, reagent grade; and diphenylamine, special indicator grade, were obtained from Fisher. All other reagents were analytical reagent grade, or the highest purity commercially available.

**Isotopes**

\[ ^{14}C \]Leucine, \[ l-[guanidino-^{14}C] \]arginine, and \[ l-[^{18}S] \]methionine were purchased from International Chemical and Nuclear Corporation, Irvine, Calif. Butyl-PDD ((4'-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxidole), primary fluor, and scintillation toluene and Biosolv (BBS-3) were products of Beckman Instruments, Inc., Fullerton, Calif.

**Clofibrate**

This was supplied in two forms which were the kind gift of Ayerst Laboratories, Inc., New York. All dietary studies were carried out using the ethyl ester; ethyl a-phenylpropionyl citrate, which was incorporated into standard Purina rat chow at the content of 0.3% (w/w) by General Biochemicals, Chagrin Falls, Ohio. In vivo studies involved the sodium salt of clofibrate, which is water soluble.

**Animals**

Male, albino Sprague-Dawley rats were employed throughout the investigation and came from Charles River Breeding Laboratories, Wilmington, Mass. The animals weighed from 150 to 200 g before being placed on the clofibrate diet. Controls were maintained on normal rat chow until they were killed.

**Apparatus**

Mitochondrial counting and sizing was initially carried out with a Coulter Counter, model B, exactly as described by Gear and Bednarek (21). Final results were obtained using a Cellscope LTH-112 (Particle Data, Inc., Elmhurst, III.) coupled to a laboratory minicomputer and oscilloscope.1

Isolation of Mitochondria

Liver mitochondria were isolated by differential centrifugation (22) from tissue homogenates in 0.25 M sucrose and stored at 0° at 50 mg of protein per ml. Heavy and light mitochondrial fractions were combined for the present studies. Preparation usually required about 1½ hours from killing the rats, and all experiments with the mitochondria were usually completed within 4 to 6 hours.

Lysosomal Isolation

A lysosomal fraction was prepared according to the procedure of Sawant et al. (23), except that to increase the final yield and speed of isolation, the final washing, and centrifugation were omitted. Usually five to seven rats were killed to obtain a final fraction sufficient in amount and concentration for the subsequent assay of neutral protease activity.

Assay of Enzymic Activities

NADP-Malate Dehydrogenase—The method described by Ochoa (24) was followed with Lubrol-WX being employed to release the enzyme completely from the mitochondrial matrix.

Cytochrome c Oxidase—This was monitored spectrophotometrically at 25° by following the oxidation of reduced cytochrome c at 550 nm (27). Lubrol-WX was added at 0.7 mg per mg of mitochondrial protein to activate fully the enzyme. Activity is expressed as a first order reaction constant k⁻¹.

Cytochrome c Reductase—NADH rotenone-sensitive, rotenone-insensitive, and succinate activities were assayed exactly as described by Sottocasa et al. (25). The rotenone concentration was 0.5 μM. Lubrol-WX was not employed since it inhibited the activities by about 20%.

Xyloglucine Hydrolase—This enzyme was assayed by the procedure given by Hayashi (26) with a Cary 14 spectrophotometer. The mitochondrial fractions were solubilized 15 min before assay by adding Lubrol-WX at a ratio of 0.3 mg per mg of mitochondrial protein, so as to minimize swelling effects.

Monoamine Oxidase—The method of Tabor et al. (27) was employed in which benzaldehyde formation is monitored spectrophotometrically at 550 nm. Lubrol-WX was employed as above, to minimize light-scattering effects.

Acid Phosphatase—This activity was assayed by measuring the amount of p-nitrophenyl phospho acid hydrolyzed at 37° (28). The specific activity of the enzyme is defined as micromoles of p-nitrophenol liberated per min per mg of protein, with an extinction coefficient of 18.8 X 10² M⁻¹ cm⁻¹ being employed.

Neutral Protease Activity—The procedure which was developed is based on that described by Alberti and Bartley (29). An important aspect of the assay is that concentrated mitochondrial suspensions must be used since proteolytic activity is not completely linear with protein concentration (Table 2, Ref. 29). Thus, duplicates, where protein concentration was 10 and 20 mg per ml, were routinely employed. In this case, the qmax, micromoles amino acid liberated per hr per mg of mitochondrial protein, is almost constant.

One result of Alberti and Bartley's research was the observation that phosphate was a powerful inhibitor of proteolysis. Consequently, the influence of phosphate was tested in all assays. The following test tubes, in duplicate, were set up 0.5 ml final volume.

<table>
<thead>
<tr>
<th>Tube</th>
<th>TES buffer, pH 7.4</th>
<th>KPO₄, pH 7.4</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td></td>
<td>2 mM</td>
<td>5 mM</td>
<td>10 mM</td>
<td>50 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Before incubation, 10 and 20 mg of mitochondrial protein were added to Tubes 0 and 1, respectively, to serve as endogenous blank, followed by 1 ml of 1.0 N perchloric acid. Then 5 and 10 mg of mitochondria were added to their respective tubes at the various phosphate concentrations, with incubation on a shacking water bath being for 1 hour at 37°. The reaction was stopped by addition of 1 ml of 1.0 N perchloric acid. Following a 15-min period at 0°, the tubes were centrifuged and the supernatants assayed for free amino acids, after neutralization and removal of the KOClO₄. The amino acids were estimated by a modified ninhydrin procedure.

The abbreviation used is: TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

The specific activity of the enzyme is defined as micromoles of p-glutamate being employed as a standard.

After correction of the incubated tubes by the appropriate dilution factors and endogenous amino acid blank, the results are expressed as a qmax, similar to those of Alberti and Bartley (29); that is, micromoles of amino acid liberated per hr per mg of mitochondrial protein.

The TES buffer selected for the assay of neutral protease activity was based on a survey of five "Goods" buffers (30). The results revealed that TES and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid were superior to the rest in maintaining incubation pH at the end of the hour, close to the original pH of 7.40. TES, however, gave the highest qmax of 0.277 μ mole per hr per mg. Triat experiments also compared NaCl and sucrose with the known inhibition caused by phosphate. It was found that 50 mM PO₄ gave about the same qmax as 250 mM NaCl and sucrose at 0.15, thereby decreasing the qmax by 50%, so the inhibitory effect is not simply a matter of toxicity.

All enzyme activities were measured at 25°, except where specially noted, with a Gilford automatic sampling spectrophotometer coupled to a Honeywell Electronic 19, 10" recorder. The exception was kynurenine hydroxylase, which was monitored with a Cary 14 spectrophotometer and Lamb Spectronic 700, with automatic sampling cuvette, was used to obtain the extinction values for the ninhydrin amino acid assay.

Respiratory Control

This was measured by a polarographic technique with the sucinylated medium described by Gear and Lehninger (31). It was routinely estimated as an index of mitochondrial structural integrity immediately following completion of the mitochondrial preparation.

Estimation of Cytochromes (a + a₃), b, c₁, and c

These were measured using the 0.1 slide wire of a Cary 14 spectrophotometer according to the technique of Williams (32), with more recently reported extinction coefficients for the appropriate wavelength pairs (33).

RNA Determination

This was done by the orcinol method of Schneider (34). The assay mixture contained orcinol dissolved immediately before use in concentrated HCl containing FeCl₃. After heating for 20 min in a boiling water bath, the mixture was allowed to cool and then read against a blank at 660 nm. Standard curves were prepared from RNA purified from Torula corresponding to 10 to 100 μg of RNA.

DNA Estimation

The dihydrolamin method according to Burton (35) was used. The assay mixture contained freshly dissolved diphenylamine in glacial acetic acid with subsequent addition of aqueous acetaldehyde. After incubating for 36 to 20 hours at 30°, extinctions at 300 nm were read against a blank, with standard curve from 20 to 200 μg of salmon sperm DNA.

Isotope Incorporation Studies

Long Term in Vivo Estimation of Mitochondrial Half-life—Each rat was injected in the tail vein with 15 μCi of L-[guanidino-¹⁴C]-arginine in about 0.5 ml of isotonic saline. In one experiment only about 1 μCi per animal was injected, due to a smaller purchase of the expensive isotope. Animals were then killed, always in pairs, about 7 μCi per animal was injected, due to a smaller purchase of the expensive isotope. Animals were then killed, always in pairs, 2 days after isotope administration, up to 20 days, so as to obtain an accurate estimation of mitochondrial half-life with this non-reutilizable isotope (36).

Short Term in Vivo Incorporation—Here, since reutilization is not a problem, 10 μCi of L-[¹⁴C]methionine were given intravenously to each rat. Injections were accurately timed so that animals could be killed at 10, 20, 30, and 60 min after injection. This time scale is based on the experience of Beattie et al. (37) who demonstrated very rapid in vivo labeling of rat liver mitochondria.

In Vitro Amino Acid Incorporation—There has been considerable discussion as to the most suitable medium, especially concerning energy supply, for the in vitro amino acid incorporation into mitochondrial protein. A medium with an ATP-generating...
system (38, 39) was chosen, and incubation conditions were at 30°
for up to 1 hour with most of the sterile precautions recommended
by others (38). The amino acid mixture, minus leucine, was 2.5
mM with respect to alanine and had the molar ratios suggested by
Kuypers et al. (40). Final mitochondrial concentration was 2.5 mg
of protein per ml and L-[U-14C]leucine was added at 0.25 μCi per
ml to initiate incorporation. The reaction was stopped at the
various time intervals with trichloroacetic acid. The precipi-
tates were washed twice with cold 10% trichloroacetic acid and
prepared for scintillation counting as described below.

Scintillation Counting
The well washed precipitates from the various amino acid
incorporation studies were dissolved in a small volume of 1 N
NaOH. The pH values of the resulting clear solutions were then
adjusted to between 7.5 and 8.5 with 2 N acetic acid and the solu-
tions diluted to give a final salt concentration of between 0.2 to
0.3 M. To 1 ml of these solutions were then added 10 ml of a
scintillation mixture containing (per liter) 8 g of butyl-PBD, and
15% of Biosolv (BBS-3). The solutions cleared immediately, but
were not counted for several hours so as to allow for phosphores-
cence decay. Counting was carried out in a Beckman LS-230
liquid scintillation counter. Results were expressed as counts
per min per mg of protein actually contained in the final, neutral-
ized solutions.

Protein Estimations
The ultraviolet absorption method of Murphy and Kies (41)
was generally used on account of its speed and sensitivity. Some-
times, however, the Folin method (42) was also employed. Bovine
serum albumin was used as reference standard.

RESULTS

Influence of Clofibrate on Liver Content of Mitochondria
Rats were given the drug at 0.3% (w/w) in their diet and mito-
chondria isolated after 2, 4, 6, 10, and 15 days. The results illus-
trated in Fig. 1 demonstrate clearly the ability of clofibrate
to induce a 100% increase in hepatic mitochondrial content within
10 days. Significance (p < 0.001) was high for all time intervals
except, at 2 days, p < 0.1. The data were derived from nine
separate experiments spread over 18 months with the same num-
ber of control animals being sacrificed on each experimental day;
the livers from two animals were always pooled before mito-
chondrial isolation. Not shown are changes in liver weight.
However, these have been extensively documented (8, 10-12, 17),
and in general for rats a 25 to 40% increase in weight occurs after
2 weeks on this diet. Also not shown are data obtained at 20
and 30 days. A slow but steady increase relative to the control
animals still occurred at these times.

The yields per animal are lower than those reported by Kurup
et al. (11), but this may reflect different rat strains. One im-
portant question to be asked was whether a change in the yield
of isolated mitochondria could be responsible for some of the
observed results. This was tested by assaying for total cyto-
chrome c oxidase activity in the nuclear, mitochondrial, and
supernatant fractions. Both diet and control animals gave es-
centially identical percentage yields in the three major fractions
(Table I). This observation makes very unlikely that differential
mitochondrial fragility is responsible for the higher net yields
obtained on clofibrate treatment. The total yield of cytochrome
c oxidase per animal (Table I) also increased essentially in pro-
portion to the recovered mitochondrial protein yield. A near
doubling in the specific activity of homogenate cytochrome c
oxidase was reported by Hess et al. in 1965 (9). In addition,
Kurup et al. (11) revealed that the specific activity of succinate
oxidase either per mg of liver protein, or per g wet weight of liver,
increased dramatically and was about twice in the latter case.
All of these results then confirm the specific increase in total liver
mitochondria.

Mitochondrial Respiratory Control
This was tested on the same preparations used for Fig. 1, and
the data are given in Table II. It may be noted that at 6 days
on clofibrate there was a significant increase (p < 0.005) in respi-
ratory control to a value of 5.52. This could well be related to
a minimum in neutral protease activity, which will be presented
later. The increase in respiratory control is reminiscent of
Kurup et al. (11), who did not present any statistical information,
as well as in a more recent study of kidney cortex mitochon-
dria (43).

Matrix and Inner and Outer Membrane Changes
It had been previously demonstrated (44) that during a period
of massive mitochondrial biogenesis such as during liver regenera-
tion, that a significant decrease in the specific activity of outer
mitochondrial membrane enzymes occurred. On the other hand,
matrix and inner membrane enzymes were constant. Interest-
ingly, when rats were placed on the 0.3% (w/w) clofibrate diet, a
similar decrease in the outer membrane enzymes, monoamine

FIG. 1. The influence of clofibrate on the mitochondrial yield
per animal. The data are derived from nine series of experiments,
with two rats being killed at each experimental period and the
number of control animals equaling the diet-treated ones.

TABLE I
Total cytochrome c oxidase activity in liver homogenates and
enzyme recovery in isolated mitochondrial fractions
Results are expressed as percentages. The total activity per
animal represents the average from two separate series with clo-
fibrate; one being direct measurement by summation of the total
nuclear, mitochondrial, and supernatant activities; and the
second from knowledge of cytochrome c oxidase specific activity
(Table III), the total recovered mitochondrial protein (Fig. 1),
and the percentage of enzyme recovery in the mitochondrial
fraction.

| Days on clofibrate | Control
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total homogenate activity (% of control)</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial yield (% total homogenate enzyme activity)</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>108</td>
</tr>
<tr>
<td>6</td>
<td>174</td>
</tr>
<tr>
<td>10</td>
<td>196</td>
</tr>
<tr>
<td>15</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>216</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td>15</td>
<td>40</td>
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<tr>
<td></td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>58</td>
</tr>
</tbody>
</table>
Respiratory control of mitochondria during clofibrate treatment

Eight separate series of experiments were carried out with two rats being killed at each time interval. Sodium succinate was the substrate (31) and the respiratory control is defined as the ratio of the rate of respiration in the presence of phosphate acceptor (State 3) to that following, or in its absence (State 4).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time (days)</th>
<th>Over-all</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>3.20 ± 1.39</td>
<td>3.66 ± 0.42</td>
</tr>
<tr>
<td>Clofibrate-treated</td>
<td>3.96 ± 1.42</td>
<td>3.82 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.1</td>
<td>p &gt; 0.1</td>
</tr>
</tbody>
</table>

Mitochondrial cytochrome content during clofibrate treatment

The contents were estimated from the differences in extinction at the appropriate wavelength pairs, between oxidized and reduced mitochondrial suspensions, as described under "Experimental Procedures." The data are expressed as a percentage of the control specific activity for each enzyme and represent the mean from two complete series of experiments.

<table>
<thead>
<tr>
<th>Time on clofibrate (days)</th>
<th>Specific cytochrome content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a + a1</td>
</tr>
<tr>
<td>Control (0)</td>
<td>0.201</td>
</tr>
<tr>
<td>2</td>
<td>0.193</td>
</tr>
<tr>
<td>5</td>
<td>0.232</td>
</tr>
<tr>
<td>7</td>
<td>0.178</td>
</tr>
<tr>
<td>0</td>
<td>0.212</td>
</tr>
<tr>
<td>12</td>
<td>0.215</td>
</tr>
<tr>
<td>15</td>
<td>0.187</td>
</tr>
<tr>
<td>20</td>
<td>0.171</td>
</tr>
</tbody>
</table>

Cytochrome Content during Clofibrate Treatment

The above results suggest that only outer membrane specific activities are changed during the large increase in liver mitochondrial content, and that inner membrane and matrix enzymes remain constant. To confirm the latter findings, a direct analysis of the respiratory chain cytochromes was carried out. The results in Table IV show that no significant variations occurred in the specific content of the various cytochromes.

Mitochondria DNA and RNA Content

It is known from early work with the regenerating rat liver system (45) and later reports (46-50), that the mitochondrial content of DNA and RNA increases several fold at 2 to 4 days after partial hepatectomy. This period preceded the large rise in liver mitochondrial content. In view of the similarities in time course, and net mitochondrial increase during liver regeneration (22, 45) and clofibrate treatment (Fig. 1), it was anticipated that an increase in the specific content of mitochondrial nucleic acids might also take place. However, the data (Table V) reveal no significant changes in the mitochondrial specific content of DNA and RNA; the former remaining close to the control value of 0.304 μg per mg of protein, and the latter remaining near 7.56 μg of RNA per mg of mitochondrial protein. The whole problem of contamination by non-mitochondrial nucleic acids in the isolated fractions has been carefully considered (48) and, based on the use of nuclease, electron microscopy, washing procedures,
which net change in mitochondrial levels could occur is via in-
the mitochondrial half-life, or decay constant, another way in
longer half-lives, near 8 to 10 days.

A non-reutilizable isotope, such as leuoine, (52), and obtained
and also where reutilization was avoided with [r4C]carbonate (51)
6 days. They are quite distinct from workers who failed to use
when the half-life for whole mitochondria varied between 4 and
[38x85]140]

The difference between the control half-life of 5.8 days was not
significantly different from that of the clofibrate-treated animals
dinio-Wlarginine and the results are presented in Table VII.

The nucleic acid content of well washed mitochondrial suspen-
sions was estimated as described under "Experimental Proced-
ures." Results represent the means of two series of experiments.

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Days on clofibrate</th>
</tr>
</thead>
</table>
| DNA . . . . 0.309 0.290 0.336 0.283 0.242 0.299 0.301 0.325
| RNA . . . . 7.56 8.74 8.33 5.67 7.38 7.67 7.62 5.13 |

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Days on diet</th>
</tr>
</thead>
</table>
| Diameter, μ . . . . 0.98 0.92 0.97 0.94 1.02 1.04
| Volume, μ³ . . . . 0.49 0.41 0.48 0.43 0.56 0.50 |

density gradient centrifugation, and marker enzymes, was shown
to be of minor significance compared to the major changes during
liver regeneration.

Mitochondrial Size

During liver regeneration it has been demonstrated that mito-
chondrial volume decreases by 50% 2 to 3 days after partial
hepatectomy (48, 49), followed by a rapid return to the control
volume. Using the resistive particle counting techniques de-
volved by Gear and Bednarek (21) as well as a newer one in-
volving a laboratory minicomputer, mitochondrial suspensions
were sized for up to 15 days on clofibrate. In contrast with the
dramatic decrease in mitochondrial size during liver regeneration,
no changes were evident (Table VI). Thus, the nucleic acid
changes and mitochondrial size differentiate clearly the two ex-
perimental situations where massive increase in liver content
takes place.

Isotope Incorporation Studies

Long Term, Half-life Estimation—The use of the non-reutilizable
guanidino group of arginine enables accurate estimates of
mitochondrial half-life to be gained (36). Three complete seri-
es of experiments, including controls, were carried out with [guan-
idino-14C]arginine and the results are presented in Table VII.
The difference between the control half-life of 5.8 days was not
significantly different from that of the clofibrate-treated animals
of 5.2 days. These values agree well with previous work (36) and
also where reutilization was avoided with [14C]carbonate (51)
when the half-life for whole mitochondria varied between 4 and
6 days. They are quite distinct from workers who failed to use
a non-reutilizable isotope, such as leucine, (52), and obtained
longer half-lives, near 8 to 10 days.

Short Term in Vivo Incorporation—Since clofibrate did not alter
the mitochondrial half-life, or decay constant, another way in
which net change in mitochondrial levels could occur is via in-
crease in the synthesis constant. Consequently, the rate of short
term protein synthesis was analyzed. [14S]Methionine was used
for this study, and rats were sacrificed at 10, 20, 30, and 60 min
after tail vein injection, since it is known that uptake is a very
fast process (37, 53). The present study with clofibrate is shown
in Fig. 3, with the control data being expressed as a percentage
of the specific radioactivity of the clofibrate-derived mitochondria.
The mean initial specific radioactivity (10-min incorporation time) in absolute terms was 245 cpm per mg
of mitochondrial protein for the drug-treated animals, and 168
cpm per mg for the controls.

Short Term in Vitro Incorporation—Many investigators have
described the in vitro ability of mitochondria to incorporate radio-
active amino acids into acid-precipitable material (38-40), but
there is discussion as to exactly what is being labeled (54). In
any event, the efficiency of in vitro labeling in mitochondria
from clofibrate-treated animals was tested, especially since mitochon-
dria from regenerating rat liver are more active at in vitro
FIG. 4. In vitro mitochondrial protein synthesis. Ten rats were placed on the clofibrate diet and then killed at 2, 6, 10, and 20 days. Two control animals were killed on each experimental day and in vitro protein synthesis with [14C]leucine was carried out as described under "Experimental Procedures." Results are expressed as counts per min of [14C]leucine per mg of mitochondrial protein, with the 0 experimental days being averaged together.

Incorporation than controls (48, 55). The results of incorporation at 0, 20, 30, and 60 min are given in Fig. 4, and show that, in sharp contrast to the in vivo situation (Fig. 3), mitochondria from clofibrate-treated animals were on the average 61.2% as efficient as their controls. The data represent the average rates of incorporation at 2, 6, 10, 15, and 20 days on clofibrate. A noteworthy observation, before averaging the data, was that there was no difference between diet and control animals at 2 days. The inhibitory effect became apparent only after this time.

Influence of Clofibrate on Mitochondrial Neutral Protease Activity

Mitochondrial neutral proteases have been described and their properties investigated (29, 56, 57), but, apart from speculation (58), no clear role has been ascribed to them. It was thought that they might be involved in mitochondrial turnover. To consider this possibility, their activity was studied during a period of massive change in liver content of mitochondria, such as between 2 and 10 days of clofibrate treatment.

When neutral protease activity was assayed in mitochondria from clofibrate-treated animals, it was tested at 2, 4, 6, 10, 15, and 20 days after initiating the diet. Four complete series of experiments were carried out, with controls, and two rats being killed at each time. Each test was run at 0, 2, 5, 10, and 50 mM phosphate to monitor the pattern of phosphate inhibition. The mean values from each of the six time intervals for increasing phosphate levels are shown in Fig. 5. The control mitochondria in the absence of phosphate is set at 100%. The absolute value for this control was 0.334 ± 0.075 pmole of amino acid produced per hour per mg of protein at 37°C. The absolute Qmax for the clofibrate-derived mitochondria was 0.206 ± 0.059 pmole (p < 0.001). These data then demonstrate the powerful in vivo inhibition that clofibrate has on the mitochondrial protease activity.

The time course during continuing clofibrate administration for protease activity is illustrated in Fig. 6. The data represent the mean percentage of inhibition from each of the phosphate concentrations. Once again, the striking inhibitory ability that clofibrate possesses at all times during drug treatment is evident, with a maximum 50% inhibition occurring at 20 days. It may be noteworthy that this was the one time the mitochondria had a highly significant increase in respiratory control (Table II).

The endogenous amino acid content of mitochondria from all the preparations used for the control neutral protease measurements was 0.0324 ± 0.009 pmole per mg of mitochondrial protein, and 0.0319 ± 0.008 pmole per mg (p > 0.1) for the mitochondria from clofibrate-treated animals.

Having obtained these results for an in vivo action of clofibrate on protease activity, it was necessary to examine whether the drug might inhibit in vitro. Thus, the sodium salt of clofibrate, which is both water-soluble and the form the drug exists in plasma, was added directly to mitochondria at various levels. The results in Fig. 7 do show that there is a slight inhibition, but no more than expected from NaCl. Consequently, it is likely that the in vivo inhibition is either the result of a biotransformation of the drug or some secondary effect through another effector, either of low or high molecular weight.
There has been some discussion whether the neutral protease activity associated with mitochondria might simply reflect residual lysosomal action at neutral pH due to the presence of contaminating lysosomes (56–58). However, the published evidence strongly supports the intrinsic mitochondrial origin of the neutral proteolytic activity. Nevertheless, to test whether lysosomal artefacts might conceivably cause the earlier results (Fig. 6), acid phosphatase activity was measured in both the original homogenates and final mitochondrial suspensions. The results (Table VIII) reveal that there was neither a change in homogenate specific activity, nor in the mitochondrial fractions, to parallel in any way the consistent and highly significant drop in neutral protease activity (Fig. 6).

A final set of experiments was carried out on the neutral protease activity of a partially purified lysosomal fraction (23), to see whether the clofibrate-sensitive activity could be of mitochondrial origin. The data in Table IX show that there is indeed activity in the lysosomal fraction. However, it is less than that seen in control mitochondria of 0.334 μmole per hr per mg of protein, and this makes it unlikely that the higher mitochondrial activity is the result of lysosomal contamination. Also it may be noted in Table IX that lysosomes from clofibrate-treated animals did not reveal a decreased neutral protease activity. If anything, activity was increased, although not significantly. Consequently, it is felt that these findings, plus the acid phosphatase levels in the mitochondrial fractions mentioned above, give weight to the validity of the clofibrate-sensitive neutral protease being truly mitochondrial.

Discussion

The results of the research reported here support a hypothesis that mitochondrial levels may be determined by the activity of their own neutral proteases. The system studied, namely, that where the hypocholesterolemic drug clofibrate stimulates a large net increase in the amount of hepatic mitochondria, revealed neutral protease activity to be consistently inhibited by about 40%. At the same time, although the mitochondrial half-life of 5 to 6 days was not influenced by the drug, the rates of short-term incorporation of radioactive amino acids in vivo were stimulated by about 50%. These findings led to the idea that a lowered activity of neutral protease could increase the amount of the most rapidly synthesized, or newly incorporated protein, in mitochondria. Previous research (37, 53, 59), has shown membrane or structural proteins to have the highest specific radioactivity within 5 min of isotope injection. It was specifically at these early times that the most stimulation in protein synthesis was seen (Fig. 3). Consequently, it is proposed that the steady state levels of these structural or membrane-binding proteins determine the final steady state levels of total mitochondrial protein.

Evidence from earlier work (37, 53, 59) has indicated that mitochondrial membrane proteins are preferentially labeled by isotopes over the bulk or matrix proteins. What is then possible is that the availability of an intrinsic binding protein(s) could regulate whether additional proteins are incorporated into the membrane. In this context it is now known that three of the subunits of cytochrome c oxidase are synthesized by mitochondria.

Table IX

Neutral protease activity of purified lysosomal fractions during clofibrate treatment

The data is derived from 2, 5, and 6 days of clofibrate treatment, and compared against three sets of controls; five to seven rats were killed for each lysosomal purification, and the livers were pooled. Neutral protease activity in the absence of any phosphate was assayed as described under "Experimental Procedures." Acid phosphatase was also monitored in both the initial liver homogenate and final lysosomal fraction to obtain an indication of the relative purification.

Table VIII

Acid phosphatase activity during clofibrate treatment, measured in both homogenates and mitochondrial suspensions

Activity was measured by the hydrolysis of p-nitrophenyl phosphate as described under "Experimental Procedures." All values are expressed as micromoles of substrates hydrolyzed per min per mg of protein.

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Control</th>
<th>Over-all mean</th>
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<tbody>
<tr>
<td></td>
<td>Time on clofibrate (days)</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.032 ± 0.005</td>
<td>0.032 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>0.041</td>
<td>0.261 ± 0.011</td>
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<tr>
<td></td>
<td>0.039</td>
<td>0.261 ± 0.011</td>
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<tr>
<td></td>
<td>0.025</td>
<td>0.261 ± 0.011</td>
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<td>0.026</td>
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<td>0.022</td>
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<tr>
<td></td>
<td>0.025</td>
<td>0.261 ± 0.011</td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.259 ± 0.023</td>
<td>0.259 ± 0.023</td>
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<tr>
<td></td>
<td>0.258</td>
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rial ribosomes (60), and in their absence the remaining three cytoplasmic subunits are not incorporated. Similarly, components of mitochondrial ATPase which are synthesized on cyto-
ribosomes will not be incorporated into the inner membrane unless the appropriate binding protein is available (61). The present investigation of mitochondrial neutral protease activity provides an experimental rationalization for the concepts just discussed. Namely, the protease may have a higher affinity for or (because of its intramitochondrial localization) will preferentially degrade those proteins being most rapidly synthesized, which include the membrane-binding proteins considered above. The effect of this for clofibrate, which inhibits, although not in vitro (Fig. 7), the mitochondrial protease, would be to raise the steady state levels of the binding protein, or proteins. An observation of Alberti and Bartley (56) is highly significant in this context. They found that when chloramphenicol inhibited in vitro mitochondrial protein synthesis, amino acid production decreased. Thus, there is a direct relationship between rate of synthesis of new protein, which in vitro is only membrane-linked (40, 54, 62), and the rate of protein breakdown. In other words, the protease preferentially acts on newly synthesized membrane protein. It is possible to conjecture that some of these proteins may be proteolipids (63), as evidenced by electrophoretic behavior and high specific radioactivity after in vitro labeling (54).

A few additional comments concerning the neutral protease activity are in order. It was mentioned under "Results," but should be emphasized again, namely, there is a variety of evidence that lysosomes, either broken or whole, do not contribute to the neutral proteolytic activity (56, 57). This conclusion was based on differential solubilization, inhibitors, protective and swelling agents, electron microscopy and, finally, Sephadex chromatography of the solubilized proteases. Also the present research (Tables VIII and IX) gives additional weight to the assumption that clofibrate-sensitive protease is mitochondrial and not lysosomal in origin. An earlier observation (29) that mitochondrial disruption increased the $q_{as}$, either when sueroos was present or when phosphate inhibition was tested, suggested that only a fraction of the total mitochondrial protein was accessible to digestion. Thus, the $q_{as}$ in vivo is likely to be less than for isolated mitochondria; and, in addition, intracellular phosphate which is about 5 mM, plus other inhibitory anions, an osmotic motive of some 320 mosmolar (29), and substrate, will all combine to lower the $q_{as}$. If one assumes it to be similar to the inhibition seen at 50 mM phosphate (Fig. 5), then it is possible to calculate how long it would take the protease to digest completely 1 mg of mitochondria. For control mitochondria a $q_{as}$ of 0.062 gives a "life-span" of 6.7 days, and for clofibrate the $q_{as}$ of 0.043 yields 9.7 days.

These values are for a turnover faster than indicated by the normal mitochondrial half-life of 4 to 6 days (51). They probably mean that mitochondrial turnover does not involve the neutral proteases, a concept supported by the results with clofibrate. However, this is not ruled out completely, and whether the proteases or lysosomes play the major role in mitochondrial turnover remains a fascinating problem.

Some recent investigations have been concerned with the in vitro effects of clofibrate on liver metabolism. For example, Panini (94) reported that the sodium salt at 1 mM inhibited State 3 respiration by 50%, and a similar study (66) revealed essentially the same basic finding. Subsequently, a more complete report (66) documented two sites of interaction with the mitochondrial respiratory chain. Finally, a very recent study suggests a highly specific inhibition action on certain mitochondrial permeases. Thus, clofibrate definitely possesses inhibitory properties toward mitochondria and thus we might rationalize why the in vivo (Fig. 4) effect on protein synthesis was the reverse of that seen in vivo (Fig. 3). An alternative, and probably more likely possibility, is that isolated mitochondria lack cytoplasmic factors such as an exchange protein (57) needed for normal protein synthesis. There is consequently a great danger of extrapolating in vivo results to the in vivo situation.

One of the original stimuli for undertaking the research on clofibrate was to determine whether a system, where there was a large net increase in liver mitochondria, behaved similarly to that seen after partial heptectomy (22, 44, 45). The time course and net mitochondrial increase are about the same in both situations, but several clear similarities and contrasts exist. Interestingly, the outer membrane enzymes decreased in specific activity in both cases (44, Fig. 2); although for regenerating liver the decrease was only temporary. Another situation in which outer membrane activity declined was after thyroxine treatment of normal rats (68). It has been proposed that one of the modes of action of clofibrate could be to displace bound thyroxin. The effect is reasonable since, in both thyroxin- and clofibrate-treated rats, glycerol phosphate dehydrogenase activity increased dramatically (9, 68) as did the outer membrane activities both decrease. However, there is considerable controversy (69) and some unexplainable differences, which make the thyromimetric basis of the effects of clofibrate difficult to support.

The research discussed in this paper thus focuses attention on the influence of clofibrate on liver mitochondria. It attempts to rationalize the specific ability of the drug to increase net levels of mitochondria in terms of an inhibitory action on mitochondrial neutral protease. The over-all concepts of how the increased amount of liver mitochondria might be related to the hypocholesterolemic and hypotriglyceremic effects of clofibrate were outlined earlier, in the general introduction.

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