CHAPTER III

RESULTS

Effects of Bezafibrate on Oxidative Phosphorylation by Isolated Rat Liver Mitochondria.

1. Effect on mitochondrial respiration with glutamate plus malate as substrates.

Typical tracings demonstrating the effect of bezafibrate on mitochondrial oxygen consumption are shown in figure 14. Curve A is the control response of the mitochondria to the additions of ADP+Pi (state 3 respiration) and DNP (state 3u respiration). Addition of ADP caused respiratory stimulation which was followed by a cut-off (transition from "state 3" to "state 4" respiration) when the added ADP had been phosphorylated to ATP. When DNP was added, the mitochondrial oxidative phosphorylation was uncoupled and the respiration was stimulated to a rate exceeding that of state 3 respiration. This respiratory stimulation proceeded until all the dissolved oxygen in the incubation medium was consumed. The RCI value calculated from curve A was 6.34 indicating the mitochondria used were the good intact ones. Curves B, C, and D show the inhibition of state 3 and state 3u

respiration evoked by 20, 50, and 100 μM bezafibrate respectively. The inhibitory effect was dose-dependent, and state 3 respiration was slightly more inhibited than state 3u. From figure 14, 100 μM bezafibrate produced 28.85 and 22.08% inhibition on states 3 and 3u respiration respectively. State 4 respiration, however, was practically unaffected by bezafibrate.

Dose-response curves of bezafibrate effect on state 4, state 3, and state 3u respiration with glutamate plus malate as substrates are shown in figure 15. States 3 and 3u respiration were inhibited by bezafibrate at concentration as low as 10 μM, and the degree of inhibition increased as the dose was raised. On the other hand, bezafibrate at concentration up to 100 μM did not significantly alter state 4 respiration.

2. Effect on mitochondrial respiration with succinate as substrate.

It is well known that glutamate plus malate donate electrons to mitochondrial respiratory chain via complex I[19,20]. In this experiment,succinate which donates electrons to Co Q via complex II was used as substrate. Figure 16 reports the dose-response curves of bezafibrate effect on state 4, state 3, and state 3u respiration with succinate as substrate. Bezafibrate, at

concentration ranging from 10 to 100 μM, did not affect mitochondrial state 4 respiration since the oxygen consumption rates were not altered significantly. However, states 3 and 3u respiratory rates were somewhat fluctuated as the concentrations of bezafibrate were increased, but the difference in oxygen consumption rates between bezafibrate and control was not statistically significant.

Thus bezafibrate was found to moderately depress mitochondrial oxidative phosphorylation supported by glutamate plus malate but had no or insignificant effect when succinate was electron donor.

3. Effect on RCI and P/O ratio.

When glutamate plus malate were electron donors, bezafibrate, from 20 μM, produced the statistically significant reduction of the RCI value as shown in table 2. The control RCI value was 6.59$\pm$0.18 and gradually decreased to 4.64$\pm$0.02 as the bezafibrate concentrations were raised to 100 μM. Bezafibrate did not significantly alter the P/O ratio at all drug concentration tested.

Effect of bezafibrate on RCI and P/O ratio when succinate was substrate is recorded in table 3. Under this condition, the RCI value was significantly decreased only when the drug was presented at rather high

concentrations (70, 80, and 100 μM). The P/O ratio was unaffected by all bezafibrate concentrations.

Factors Influencing The Effect of Bezafibrate on Oxidative Phosphorylation by Isolated Rat Liver Mitochondria.

1. Effect of pH.

As decribed earlier, bezafibrate is a weak acid with an ionizable carboxylic group. Thus, this chemical can exist as ionized and unionized forms, the relative concentration of which depends on the pH of incubation medium. In this experiment, the pH of reaction mixture were adjusted to 6.8, 7.2, and 7.6. The results showing the influence of pH on the bezafibrate-induced inhibition of states 3 and 3u respiration are recorded in table 4. It is seen that bezafibrate, at 40 and 100 μM, produced less inhibition on both states 3 and 3u respiration as the pH increased from 6.8 to 7.6. However, significant inhibition was observed at high dose level (100 μM) and, particularly, at pH 7.6. State 4 respiration (not shown) was not altered by bezafibrate at three different pH tested.

2. Effect of dithiothreitol (DTT).

DTT, a sulfhydryl-protecting substance, was

introduced to this experiment inorder to investigate whether bezafibrate acts by combining with mitochondrial sulfhydryl group. As shown in table 5, 1.05 mM DTT did not affect mitochondrial oxidative phosphorylation, and this reagent completely failed to alleviate the inhibitory effect of bezafibrate on both states 3 and 3u respiration. It should be mentioned that the concentration of DTT employed in this study is sufficient to effectively antagonize the effect of DTNB, a sulfhydryl-blocking compound, on mitochondrial energy metabolism.

3. Effect of Mg^{2+}.

Mg^{2+} is the major divalent cation in the cytosol and also serves as the important cofactor of many cellular biochemical reactions. In this study, the concentration of Mg^{2+} in the incubation medium was varied from 0 to 9.42 mM. As reported in table 6, the inhibited states 3 and 3u respiration produced by bezafibrate was not significantly altered by varying the Mg^{2+} concentrations except one instance, i.e., 40 μM bezafibrate and 9.42 mM Mg^{2+}. Since omission of Mg^{2+} did not significantly affect the inhibitory effect, this suggested that extramitochondrial Mg^{2+} was unnecessary for bezafibrate to inhibit mitochondrial respiration.

4. Effect of bovine serum albumin (BSA)

Bezafibrate as well as many other drugs bind to serum albumin. It is therefore interesting to study whether BSA can attenuate bezafibrate effect by binding to and thereby removing the drug from the mitochondria. The study on state 3u respiration was omitted because BSA forms complex with DNP and inhibits its uncoupling activity. It is seen from table 7 that 20 mg BSA had practically no effect on state 3 respiration whereas 100 μM bezafibrate decreased the rate from 199.82±5.10 to 148.79 ng-atoms O/min/mg protein. Addition of 5, 10, or 20 mg BSA could substantially antagonize the inhibitory effects of bezafibrate on state 3 respiration. Evidently, the action of BSA was dose-related, and significant antagonism was observed with all three BSA doses.

Effect of Bezafibrate on Calcium-Stimulated Respiration by Isolated Rat Liver Mitochondria.

When Ca^{2+} was added to respiring mitochondria, it is accumulated by the mitochondria and simultaneously stimulates mitochondrial oxygen consumption. Effect of bezafibrate on calcium-stimulated respiration with glutamate plus malate as substrates is shown in figure 17. Curve A shows the control respiratory response of rat liver mitochondria to $CaCl_2$. Addition of $CaCl_2$ caused

respiratory stimulation which was followed by a cut-off when practically all of the added Ca^{2+} had been transported into the mitochondria. Curves B, C, and D show the inhibitory effect of 20, 40, and 100 μM bezafibrate on oxygen consumption rate during calcium-stimulation respectively. Percent inhibition observed with 100 μM bezafibrate was approximately 25% (curve D). The inhibition was dose-dependent as shown in the dose-response curve (figure 18). The oxygen consumption rate gradually decreased with increasing bezafibrate concentration. Significant inhibition was observed with the concentration of bezafibrate as low as 10 μM.

Effect of Bezafibrate on Calcium Transport by Isolated Rat Liver Mitochondria.

The results presented in the preceding section indicated that bezafibrate inhibited mitochondrial Ca^{2+} accumulation. Further experiments were then performed with calcium-selective electrode to determine directly calcium transport across mitochondrial inner membrane.

1. Effect on substrate-supported calcium transport.

Figure 19 shows the effect of bezafibrate on mitochondrial calcium uptake (left panel) and release (right panel) with glutamate plus malate as substrates.

In controls (curve a, both panels), calcium was rapidly taken up by the mitochondria and the accumulated calcium was well retained during 10 min incubation period. In the left panel, when 50 and 100 μM bezafibrate were initially present in the medium (curves b and c respectively) calcium uptake was rather slightly reduced. However the accumulated calcium could not be retained by the mitochondria, and was completely released subsequently. The calcium-releasing effect was more rapid in onset and rate with higher drug concentration (100 μM). Curve d shows the effect of 10 μg rotenone, the site I respiratory chain inhibitor, present initially which completely inhibited calcium uptake by the mitochondria.

In the right panel, similar calcium-releasing effect was also observed when bezafibrate was added after the mitochondria were allowed to take up calcium for 5 min (curves b and c). Curves d and e show the calcium-releasing effect of the uncoupler, DNP, and the respiratory chain inhibitor, rotenone, respectively. Compared with bezafibrate, the calcium efflux rate was most rapid with DNP while rotenone produced an initial rapid followed by a slower rate of calcium extrusion. The three drugs practically released all of the accumulated calcium by the mitochondria.

2. Effect on ATP-supported calcium transport.

The effect of bezafibrate on ATP-supported mitochondrial calcium transport is shown in figure 20. In the left panel, calcium uptake was slightly inhibited by 50 and 100 μM bezafibrate (curves b and c respectively) when compared with the control (curve a). The releasing effect also occured but was small with 50 μM bezafibrate whereas 100 μM bezafibrate caused much more rapid and nearly complete release.

The right panel shows the bezafibrate (curves b and c) - and DNP (curve d) - induced calcium release compared with control (curve a). The releasing effect exerted by 50 μM bezafibrate was small compared with 100 μM bezafibrate and DNP. Neither drugs caused complete calcium release during 15 min incubation period.

3. Effect of bezafibrate on mitochondrial calcium transport compared with ruthenium red.

It is generally accepted that mitochondrial calcium uptake is mediated via a calcium uniporter which is specifically blocked by ruthenium red. Experimental results comparing the effects of bezafibrate and ruthenium red on mitochondrial calcium transport are depicted in figure 21. In these experiments glutamate plus malate

were respiratory substrates. In the left panel, bezafibrate added initially has only minor inhibitory effect on calcium uptake but exerted strong calcium-releasing effect subsequently (curve b) as compared with control (curve a). In contrast, mitochondrial calcium uptake was totally inhibited by 3 μg ruthenium red present initially (curve c).

In the right panel, bezafibrate and ruthenium red, added 5 min after calcium accumulation by the mitochondria, produced the calcium-releasing effect (curves b and c respectively). The bezafibrate-induced calcium release was, however, complete whereas the ruthenium red-induced was not, when observed during the same experimental period.

Effect of Bezafibrate on ATPase Activity of Rat Liver Mitochondria.

In order to investigate whether bezafibrate inhibits oxidative phosphorylation by interfering with the mitochondrial ATP synthase, the effect of bezafibrate on mitochondrial ATPase activity was studied. The ATPase reaction is generally believed to represent the reversed process of the ATP synthase reaction [30]. Agents which inhibit ATP synthesis, for example oligomycin, also depress the uncoupler-induced ATPase activity. The effect

of bezafibrate on mitochondrial ATPase activity with and without DNP is presented in table 8.

In the absence of DNP, the control ATPase activity was very low since the enzyme functions in the direction of ATP synthesis. Bezafibrate at 50 and 100 μM slightly enhanced the enzyme activity. The stimulation was significant with high dose (100 μM). Oligomycin, at concentration which completely blocks state 3 respiration, not only failed to inhibit but further stimulated the bezafibrate-activated mitochondrial ATPase activity.

When 0.1 mM DNP was present, the ATPase activity greatly increased due to the energy-dissipating effect of the uncoupler. As usual, the DNP-activated ATPase activity was severely inhibited by oligomycin. Bezafibrate at 50 and 100 μM caused small but significant inhibition of the enzyme activity which was maximally stimulated by DNP.

Effect of Bezafibrate on Mitochondrial Monoamine Oxidase (MAO)Activity.

The effect of bezafibrate on MAO activity by isolated rat liver mitochondria is shown in figure 22. In this study, benzylamine was the substrate for the enzyme MAO. Rotenone was first added to prevent oxygen consumption due to oxidation of endogenous substrates.

Curve A is the control response of mitochondrial MAO to the addition of benzylamine. The initial rate of oxygen consumption was slow, but when benzylamine was added the rate increased threefold(from 8.34 to 25.86 ng-atoms O/ml/min). Pargyline, a MAO inhibitor, completely blocked the increase in oxygen consumption induced by benzylamine (curve B). Bezafibrate, at 50 and 100 μM, had no effect on the benzylamine-induced increase in oxygen uptake indicating that MAO activity was unaffected by the drug at concentrations employed(curves C and D).

Comparison of The Effects of Bezafibrate and Clofibric Acid on Oxidative Phosphorylation and Calcium Transport by Isolated Rat Liver Mitochondria.

Clofibric acid, the active form of clofibrate, was used in this study to compared with bezafibrate action on the main bioenergetic functions of mitochondria.

1. Effect on oxidative phosphorylation.

In figure 23, curve A is the control response of mitochondria to the addition of ADP+Pi and DNP. Curve B shows the inhibitory effect of 100 μM bezafibrate on states 3 and 3u respiration. Note that state 4 respiration was not altered by bezafibrate. The effect of 400 μM clofibric acid is shown in curve C. Clofibric acid

stimulated state 4 respiration and, similar to bezafibrate, inhibited states 3 and 3u respiration. It can also be seen from table 9 that state 4 respiration was significantly stimulated by 400 μM clofibric acid while 100 μM bezafibrate had no effect. Moreover, bezafibrate appeared to inhibit state 3 stronger than state 3u respiration whereas the opposite was observed with clofibric acid.

Table 10 shows the effect of bezafibrate and clofibric acid on RCI and P/O ratio. Both drugs significantly decreased the RCI values but did not significantly alter the P/O ratio.

2. Effect on calcium transport.

The effects of bezafibrate and clofibric acid on mitochondrial calcium transport are depicted in figure 24. Left panel shows effects on mitochondrial calcium uptake. Curve a is the control in which the added calcium was rapidly taken up by the mitochondria and the accumulated calcium was well retained during 10 min incubation period. Curves b and c show the effects of 100 μM bezafibrate and 400 μM clofibric acid, presented intially, respectively. It is seen that the main effects of both drugs were to enhance calcium release from the mitochondria. In this respect, bezafibrate was evidently

more active than clofibric acid.

The effect on mitochondrial calcium release is recorded in the right panel. The results clearly confirmed that both drugs stimulated mitochondrial calcium efflux and that clofibric acid was less potent.

FIGURES AND TABLES

Figure 14. Tracings illustrating the inhibitory effect of bezafibrate on state 3 and state 3u respiration of rat liver mitochondria with glutamate plus malate as substrates.

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP+0.63 mM Pi, 0.05 mM DNP, and bezafibrate as indicated. The mitochondrial protein was 2.04 mg/ml. Total volume 1.91 ml. Temperature 37°C. The figures in parentheses are rates of oxygen consumption in ng-atoms O/ml/min.

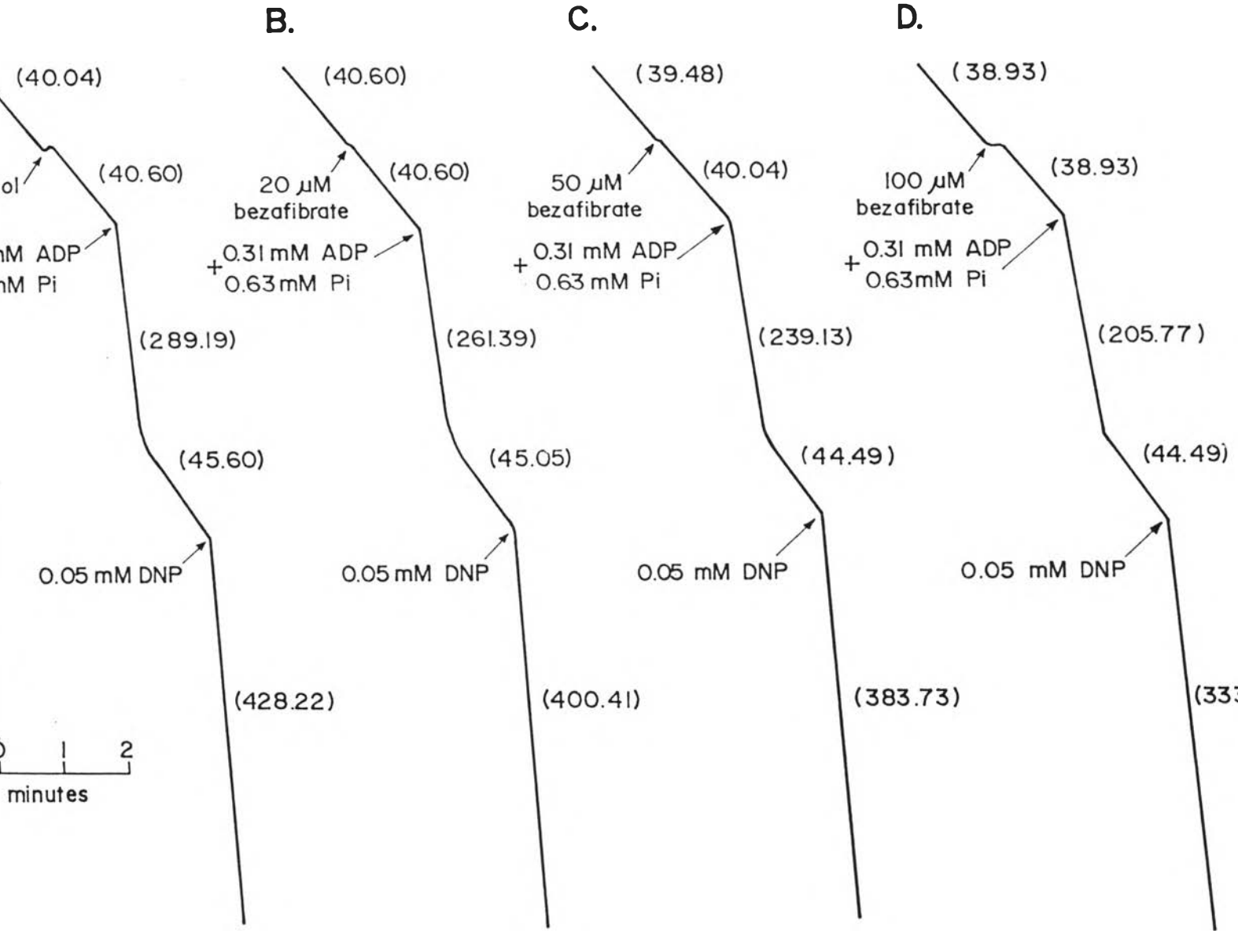

B.
C.
D.
(40.04)
(40.60)
(289.19)
(45.60)
0.05 mM DNP
(428.22)
(40.60)
20 μM
bezafibrate
(40.60)
+0.31 mM ADP
0.63 mM Pi
(261.39)
(45.05)
0.05 mM DNP
(400.41)
(39.48)
50 μM
bezafibrate
(40.04)
+0.31 mM ADP
0.63 mM Pi
(239.13)
(44.49)
0.05 mM DNP
(383.73)
(38.93)
100 μM
bezafibrate
(38.93)
+0.31 mM ADP
0.63mM Pi
(205.77)
(44.49)
0.05 mM DNP
1
2
minutes

Figure 15. The dose-response curves of bezafibrate effect on state 4 state 3 and state 3u respiration of rat liver mitochondria with glutamate plus malate as substrates.

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP and bezafibrate as indicated. The average mitochondrial protein was 2.14 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP+Pi were added 1 min after bezafibrate and DNP added during state 4 respiration. Each point represents a mean ± SEM from four experiments.

a - $p < 0.05$

b - $p < 0.01$ compared with control (no bezafibrate)

c - $p < 0.005$

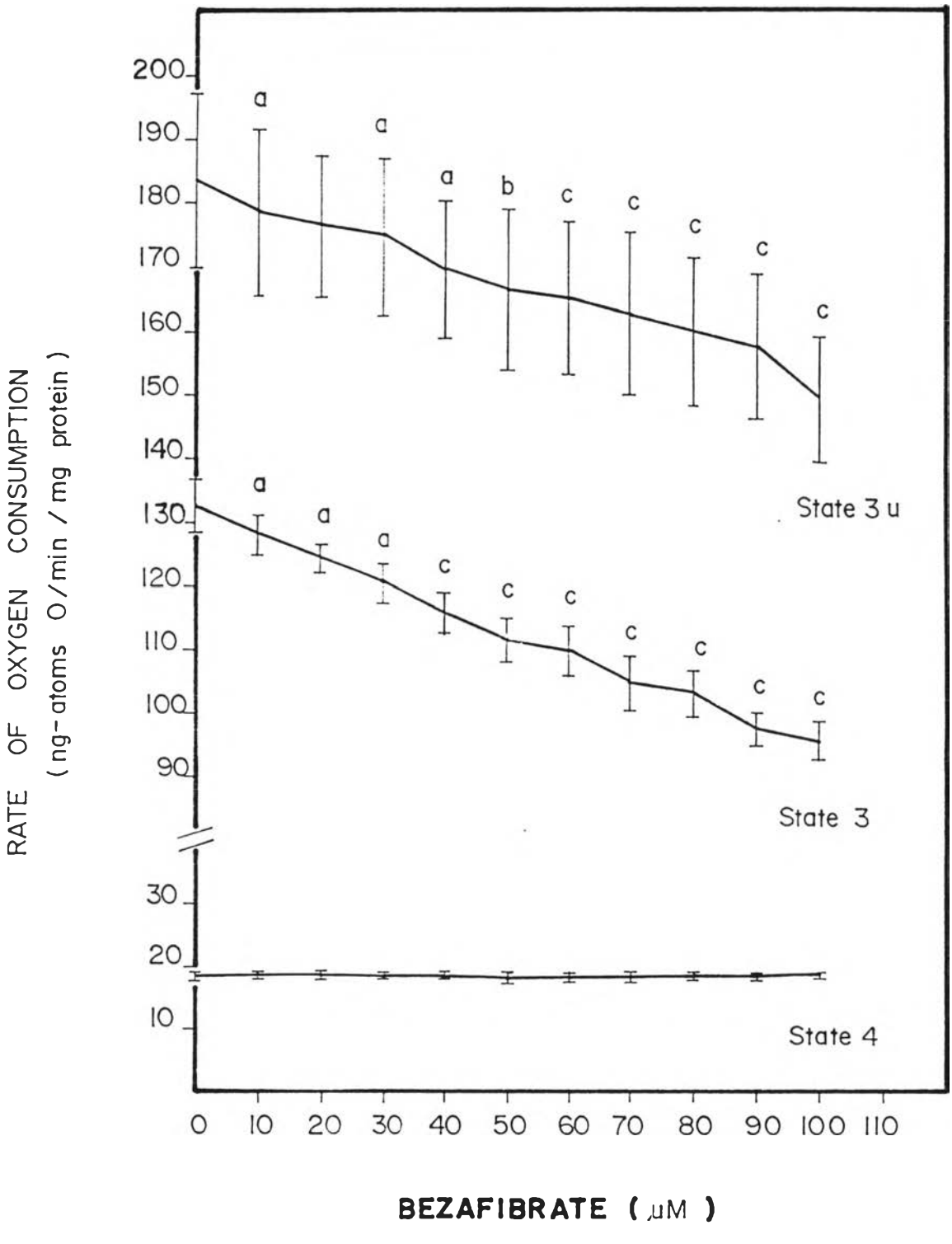

RATE OF OXYGEN CONSUMPTION
(ng-atoms O/min / mg protein)
200
190
180
170
160
150
140
130
120
110
100
90
30
20
10
a
a
a
b
c
c
c
c
c
a
a
a
c
c
c
c
c
c
State 3 u
State 3
State 4
0
10
20
30
40
50
60
70
80
90
100
110
BEZAFIBRATE (μM)

Figure 16. The dose-response curves of bezafibrate effect on state 4, state 3, and state 3u respiration of rat liver mitochondria with succinate as substrate.

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium succinate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP and bezafibrate as indicated. The average mitochondrial protein was 1.93 mg/ml. Total volume 1.91 ml. Temperature 37^{o}C. ADP+Pi were added 1 min after bezafibrate and DNP added during state 4 respiration. Each point represents a mean$\pm$SEM.

a - $p < 0.05$

b - $p < 0.01$ compared with control (no bezafibrate)

c - $p < 0.005$

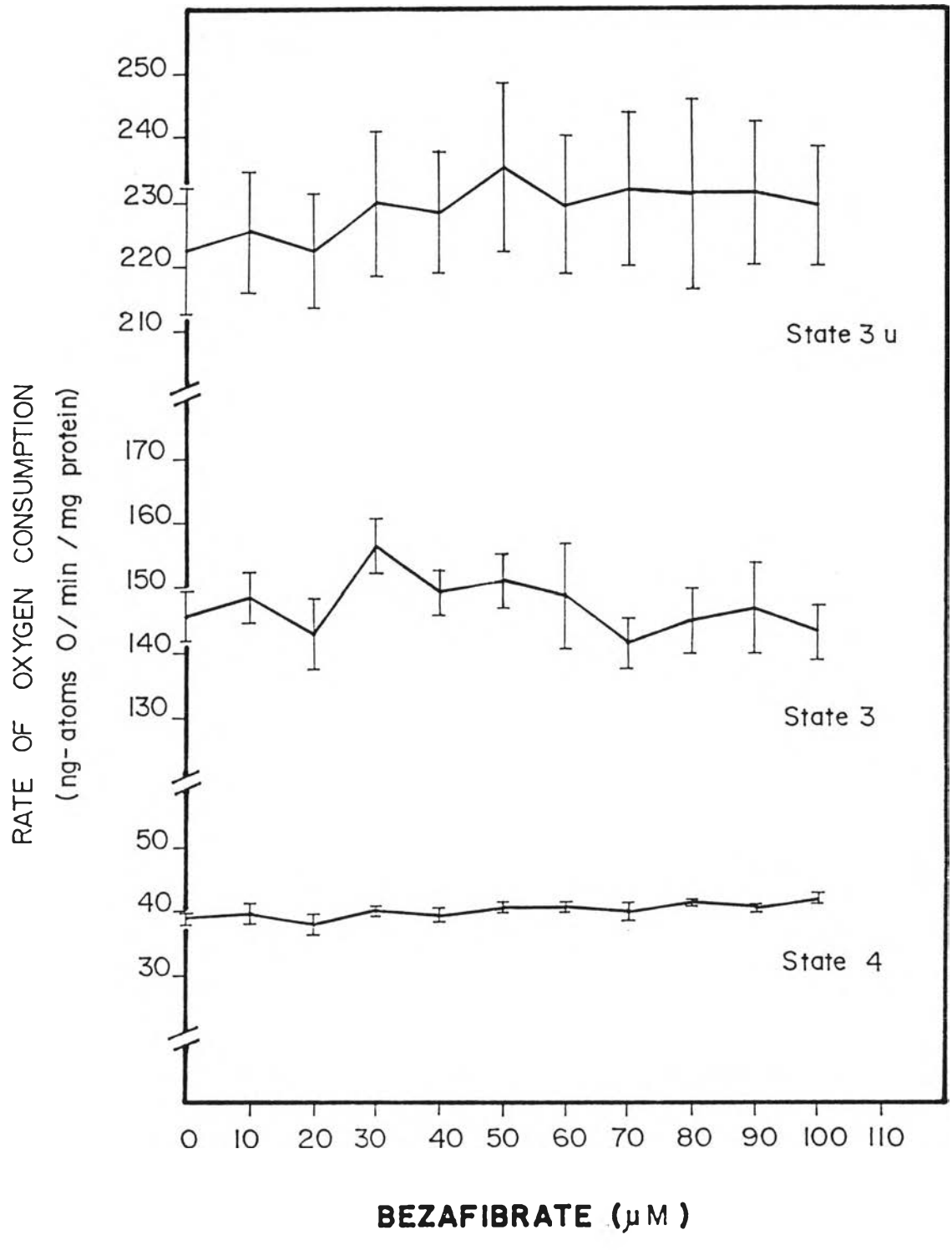

RATE OF OXYGEN CONSUMPTION
(ng-atoms O / min / mg protein)
250
240
230
220
210
State 3 u
170
160
150
140
130
State 3
50
40
30
State 4
0
10
20
30
40
50
60
70
80
90
100
110
BEZAFIBRATE (μM)

Table 2. Effect of bezafibrate on respiratory control index (RCI) and P/O ratio of rat liver mitochondria with glutamate plus malate as substrates.

Bezafibrate (μM)	RCI	P/O
0(control)	6.59±0.18	2.78±0.05
10	6.47±0.27	2.79±0.02
20	6.11±0.18[a]	2.86±0.05
30	5.87±0.10[a]	2.87±0.04
40	5.67±0.09[a]	2.89±0.03
50	5.58±0.14[a]	2.90±0.06
60	5.31±0.12[a]	3.01±0.08
70	5.02±0.05[a]	2.86±0.09
80	5.00±0.05[a]	2.94±0.09
90	4.74±0.09[a]	2.99±0.08
100	4.64±0.02[a]	2.95±0.11

a - $p < 0.05$ compared with control

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, and bezafibrate as indicated. The average mitochondrial protein was 2.14 mg/ml. Total volume 1.91 ml. Temperature 37°C. The mitochondria were preincubated with bezafibrate for 1

min before ADP+Pi were added. The RCI values and P/O ratios are expressed in means±SEM from four experiments.

Table 3. Effect of bezafibrate on respiratory control index (RCI) and P/O ratio of rat liver mitochondria with succinate as substrate.

Bezafibrate (μM)	RCI	P/O
0(control)	3.69±0.09	1.98±0.09
10	3.78±0.14	1.94±0.13
20	3.69±0.08	1.98±0.16
30	3.92±0.16	2.00±0.12
40	3.78±0.16	1.96±0.11
50	3.65±0.09	1.94±0.13
60	3.60±0.15	2.02±0.15
70	3.50±0.05[a]	2.05±0.14
80	3.51±0.09[a]	1.99±0.15
90	3.53±0.17	2.01±0.09
100	3.42±0.12[a]	2.01±0.15

a - $p < 0.05$ compared with control

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2,1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium succinate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, and bezafibrate as indicated. The average mitochondrial protein was 1.93 mg/ml. Total volume 1.91 ml. Temperature 37°C. The mitochondria were preincubated with bezafibrate for 1 min before ADP+Pi were added. The RCI

values and P/O ratios are expressed in means+SEM from four experiments.

Table 4. Effect of pH on the inhibition of state 3 and state 3u respiration by bezafibrate.

pH	Bezafibrate (μM)	% inhibition of state 3 respiration	% inhibition of state 3u respiration
6.8	40	17.22+2.97	18.18±3.89
	100	31.38±0.71[a]	27.76±2.70
7.2	40	14.94+2.46	12.69±2.98
	100	23.90±2.56	19.91±1.84
7.6	40	5.28+2.54	6.65±2.6
	100	14.89±2.45[a,b]	12.05±1.92[a,b]

a - $p < 0.05$ compared with pH 7.2

b - $p < 0.05$ compared with pH 6.8

Composition of reaction system: 37.70 mM HEPES buffer pH 6.8, 7.2 and 7.6, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, and bezafibrate as indicated. The average mitochondrial protein was 2.18 mg/ml. Total volume 1.91 ml. Temperature 37^{o}C. ADP+Pi were added 1 min after bezafibrate and DNP added during state 4 respiration. Percent inhibition of state 3 and state 3u respiration are expressed in means±SEM

from four experiments. The control state 3 and state 3u respiratory rates when incubation medium pH were 6.8, 7.2 and 7.6 were 99.78±6.71 and 118.50±17.26, 115.20±5.63 and 172.95±9.15, and 104.08±3.62 and 168.89±7.85 ng-atoms O/min/mg protein respectively.

Table 5. Effect of dithiothreitol (DTT) on the inhibition of state 3 and state 3u respiration by bezafibrate.

Experiments	Rates of oxygen consumption (ng-atoms O/min/mg protein)	
	State 3	State 3u
Control	197.45±14.52	242.06±14.04
1.05 mM DTT	201.61±14.73[a]	241.32±11.29[a]
100 μM bezafibrate	145.58± 8.76	197.46± 8.55
1.05 mM DTT + 100 μM bezafibrate	147.29±8.09[b]	190.42±5.86[b]

a - $p > 0.05$ compared with control

b - $p > 0.05$ compared with 100 μM bezafibrate

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, 1.05 mM DTT, and bezafibrate as indicated. The average mitochondrial protein was 2.56 mg/ml. Total volume 1.91 ml. Temperature 37°C. Bezafibrate was added 1 min after DTT; ADP + Pi were added 1 min after bezafibrate. DNP was added during state 4 respiration. The rates of oxygen consumption are expressed in means+SEM from four experiments.

Table 6. Effect of Mg^{2+} on the inhibition of state 3 and state 3u respiration by bezafibrate.

Mg^{2+} (mM)	Bezafibrate (μM)	% inhibition of state 3 respiration	% inhibition of state 3u respiration
0	40	9.18±1.96	10.15±1.37
	100	24.88±2.38	22.72±1.05
1.88	40	12.30±1.74	11.26±1.26
	100	25.52±1.84	19.89±0.98
4.71	40	10.23±0.97	10.73±1.93
	100	21.55±0.70	19.26±1.72
9.42	40	10.21±1.84[a]	8.83±2.19
	100	23.64±2.93	14.47±1.90

a - $p < 0.05$ compared with 1.88 mM Mg^{2+}

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, and bezafibrate as indicated. The concentrations of $MgCl_2$ and KCl were:

0 mM $MgCl_2$ and 89.53 mM KCl or

1.88 mM $MgCl_2$ and 86.70 mM KCl or

4.71 mM $MgCl_2$ and 82.89 mM KCl or

9.42 mM $MgCl_2$ and 75.79 mM KCl

The average mitochondrial protein was 1.60 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP + Pi were added 1 min after bezafibrate and DNP added during state 4 respiration. Percent inhibition of state 3 and state 3u respiration are expressed in means±SEM from four experiments. The control state 3 and state 3u respiratory rates when Mg^{2+} concentrations were 0, 1.88, 4.71 and 9.42 mM were 143.31±3.05 and 192.36±10.30, 143.07±2.13 and 195.02±9.19, 134.61±2.32 and 202.57±9.81, and 132.00±1.48 and 209.91±9.14 ng-atoms O/min/mg protein respectively.

Table 7. Attenuation of the bezafibrate-induced inhibitory effect on state 3 respiration by bovine serum albumin (BSA).

Experiments	Rates of state 3 respiration (ng-atoms O/min/mg protein)
Control	199.82±5.10
20 mg BSA	194.45±5.38
100 μM bezafibrate	148.79±1.69
5 mg BSA + 100 μM bezafibrate	160.98±0.93[a]
10 mg BSA + 100 μM bezafibrate	169.38±4.48[a]
20 mg BSA + 100 μM bezafibrate	182.91±4.34[a]

a - $p < 0.05$ compared with 100 μM bezafibrate

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP+ 0.63 mM Pi,bezafibrate and BSA as indicated. The average mitochondrial protein was 1.26 mg/ml. Total volume 1.91 ml. Temperature 37°C. Bezafibrate was added 1 min after BSA and ADP+ Pi added 1 min after bezafibrate. State 3 respiratory rates are means±SEM from four experiments.

Figure 17. Tracings demonstrating the inhibitory effect of bezafibrate on calcium-stimulated respiration of rat liver mitochondria.

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 0.94 mM potassium phosphate, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate,0.42 mM $CaCl_2$, and bezafibrate as indicated. The mitochondrial protein was 2.12 mg/ml. Total volume 1.91 ml. Temperature 37^{o}C. The figures in parentheses denote rates of oxygen consumption in ng-atoms O/ml/min.

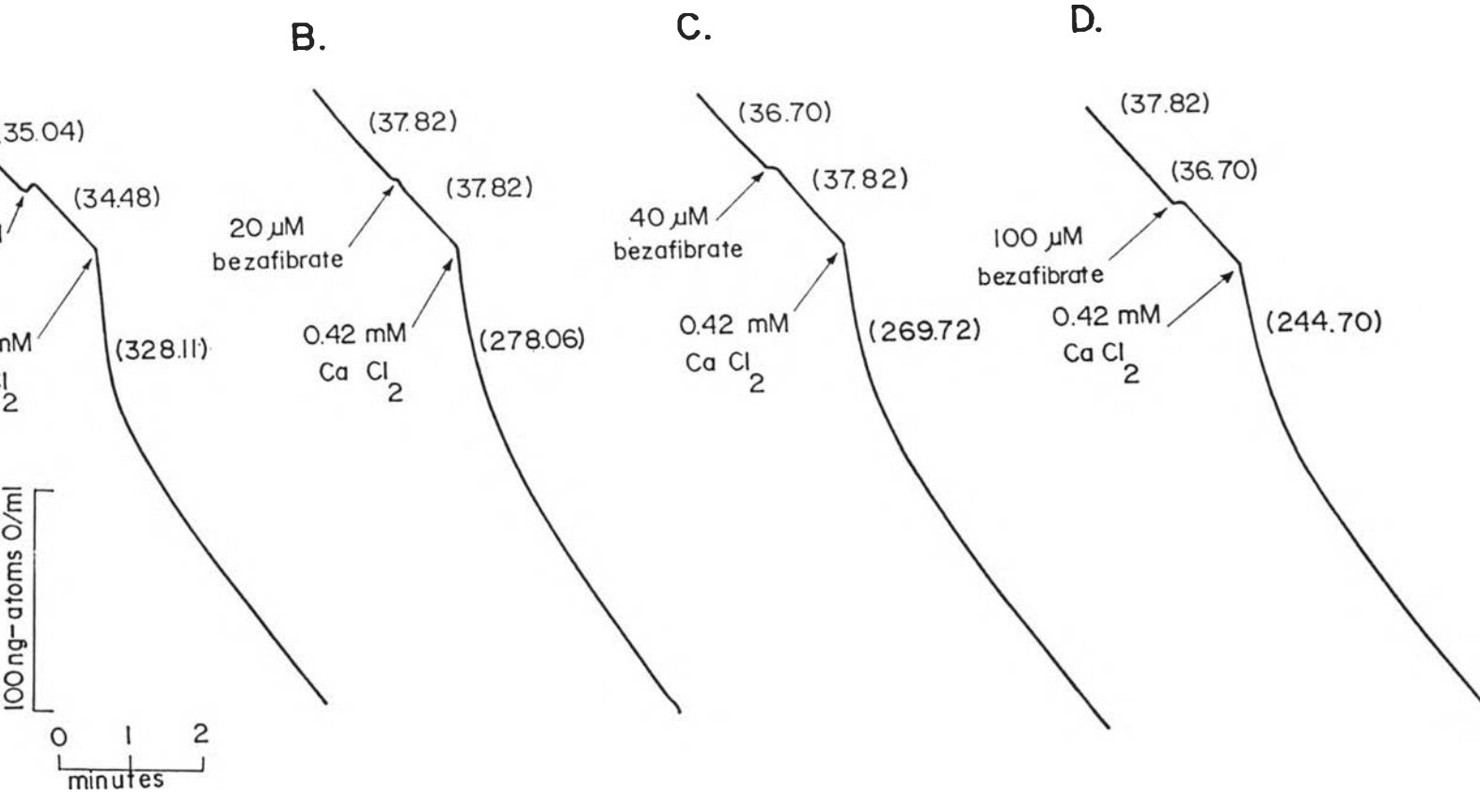

B.
C.
D.
(35.04)
(34.48)
(328.11)
nM
Ca Cl 2
(37.82)
(37.82)
20 μM
bezafibrate
0.42 mM
Ca Cl 2
(278.06)
(36.70)
(37.82)
40 μM
bezafibrate
0.42 mM
Ca Cl 2
(269.72)
(37.82)
(36.70)
100 μM
bezafibrate
0.42 mM
Ca Cl 2
(244.70)
100 ng-atoms O/ml
0
1
2
minutes

Figure 18. The dose-response curve of bezafibrate inhibition on calcium-stimulated respiration of rat liver mitochondria.

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 0.94 mM potassium phosphate, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.42 mM $CaCl_2$, and bezafibrate as indicated. The average mitochondrial protein was 1.82 mg/ml. Total volume 1.91 ml. Temperature 37°C. The mitochondria were preincubated with bezafibrate for 1 min before $CaCl_2$ was added. Each point represents mean±SEM from four experiments.

a - $p < 0.005$ compared with control.

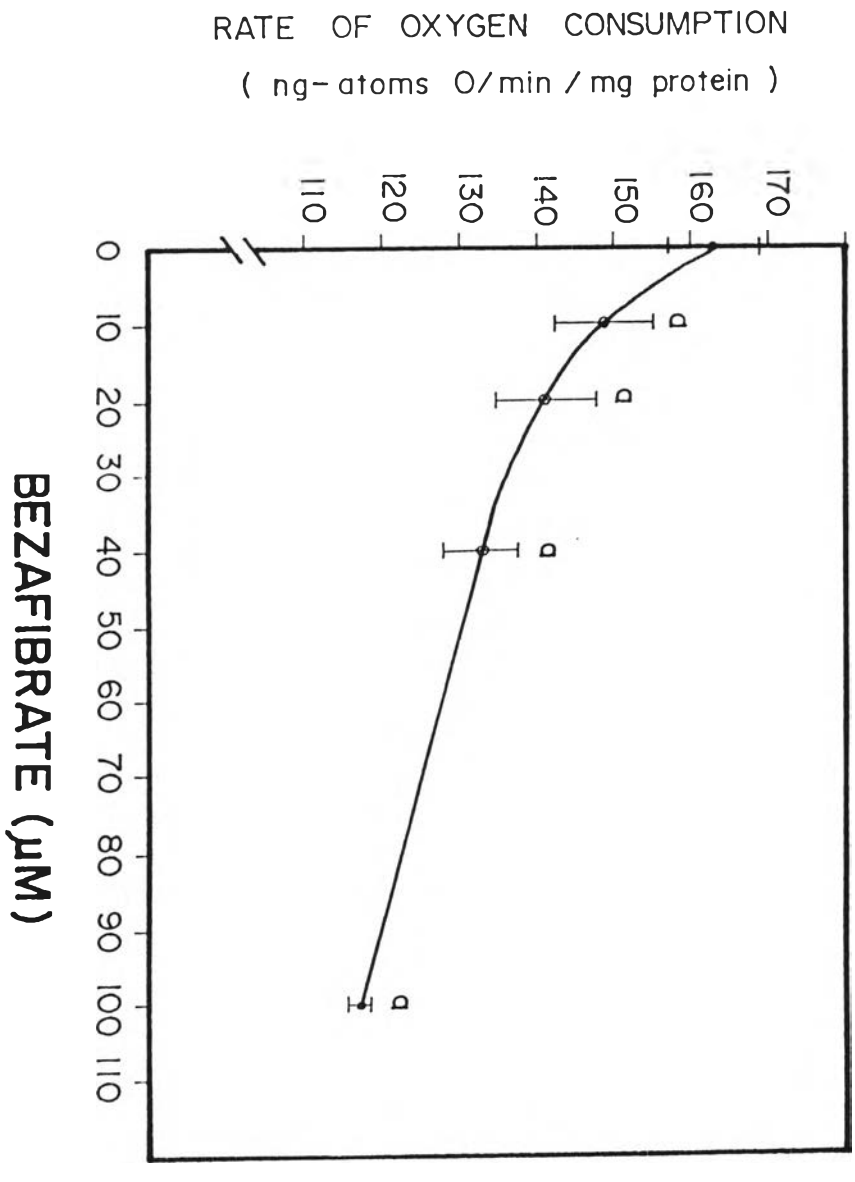

RATE OF OXYGEN CONSUMPTION
(ng-atoms O/min / mg protein)
110
120
130
140
150
160
170
0
10
20
30
40
50
60
70
80
90
100
110
BEZAFIBRATE (μM)
a
a
a
a

Figure 19. Effect of bezafibrate on substrate-supported calcium transport by rat liver mitochondria.

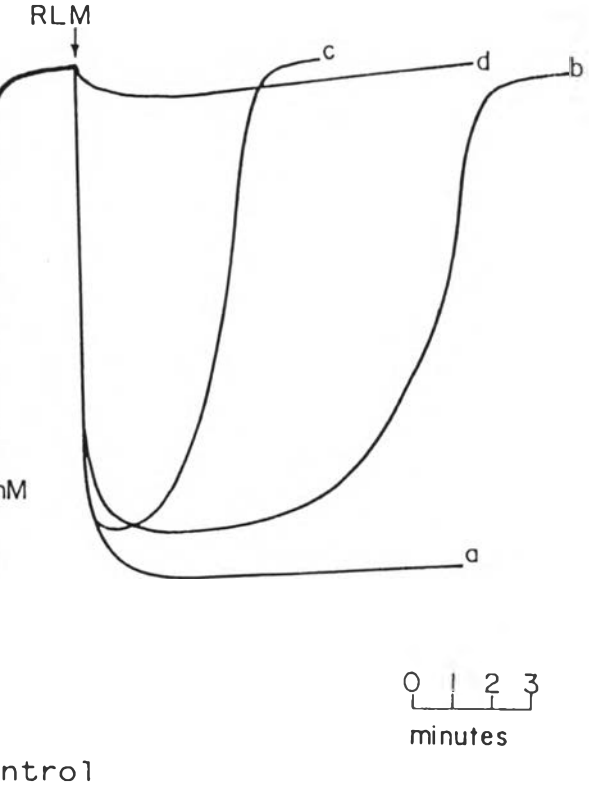
Left panel : Effect on mitochondrial calcium uptake. Composition of reaction system: 36.96 mM HEPES buffer pH 7.2, 1.85 mM $MgCl_2$, 85.02 mM KCl, 0.17 mM potassium phosphate, 16.50 mM sucrose, 4.95 mM potassium glutamate, 4.95 mM potassium malate, and 0.13 mM $CaCl_2$. Absolute ethanol, bezafibrate, and rotenone were added initially. The mitochondrial protein was 1.78 mg/ml. Total volume 3.03 ml.

Right panel :Effect on mitochondrial calcium release. Composition of reaction system: 36.96 mM HEPES buffer pH 7.2, 1.85 mM $MgCl_2$, 85.02 mM KCl, 0.17 mM potassium phosphate, 16.50 mM sucrose, 4.95 mM potassium glutamate, 4.95 mM potassium malate, and 0.13 mM $CaCl_2$. Absolute ethanol, bezafibrate, DNP, and rotenone were added 5 min after mitochondria. The mitochondrial potein was 1.78 mg /ml. Total volume 3.03 ml.

In both panels: $CaCl_2$ was first added to calibrate the calcium-selective electrode. The distance of the upward deflection following $CaCl_2$ addition denotes the concentration in the medium of added calcium ion, i.e. 0.13 mM. Calcium transport was initiated by adding the mitochondria (RLM). The upward and downward deflections

indicate the increase and decrease of calcium ion concentration in the reaction mixtures respectively.

CALCIUM UPTAKE



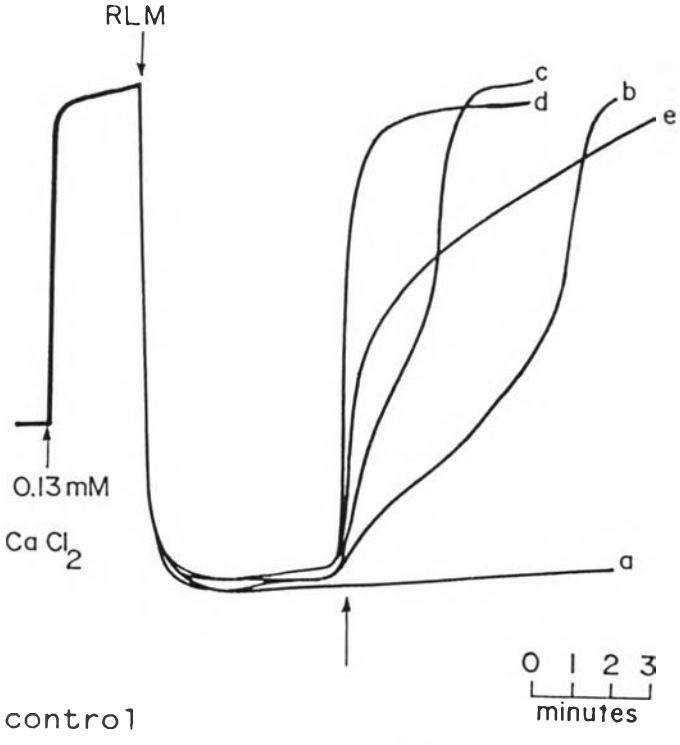
ntrol

μM bezafibrate

0 μM bezafibrate } added initially

μg rotenone

CALCIUM RELEASE



a - control

b - 50 μM bezafibrate

c - 100 μM bezafibrate } added 5 min afte

d - 0.05 mM DNP RLM

e - 10 μg rotenone

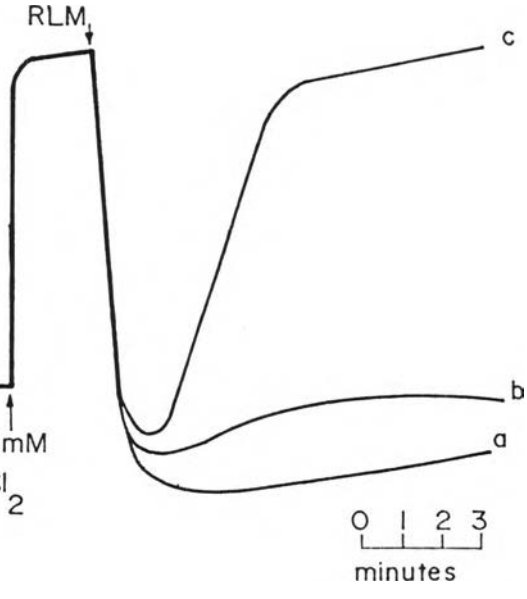
Figure 20. Effect of bezafibrate on ATP-supported calcium transport by rat liver mitochondria.

Left panel : Effect on mitochondrial calcium uptake. Composition of reaction system: 36.01 mM HEPES buffer pH 7.2, 1.80 mM $MgCl_2$, 82.83 mM KCl, 0.16 mM potassium phosphate, 16.08 mM sucrose, 2.89 mM ATP and 0.13 mM $CaCl_2$. Absolute ethanol and bezafibrate were added initially. The mitochondrial protein was 1.74 mg/ml. Total volume 3.11 ml.

Right panel :Effect on mitochondrial calcium release. Composition of reaction system: 36.60 mM HEPES buffer pH 7.2, 1.83 mM $MgCl_2$, 84.18 mM KCl, 0.16 mM potassium phosphate, 12.25 mM sucrose, 2.94 mM ATP, and 0.13 mM $CaCl_2$. Absolute ethanol and bezafibrate were added 5 min after mitochondria. The mitochondrial protein was 1.38 mg/ml. Total volume 3.06 ml.

In both panels: $CaCl_2$ was first added to calibrate the calcium-selective electrode. The distance of the upward deflection following $CaCl_2$ addition denotes the concentration in the medium of added calcium ion, i.e. 0.13 mM. Calcium transport was initiated by adding the mitochondria (RLM). The upward and downward deflections indicate the increase and decrease of calcium ion concentration in the reaction mixtures respectively.

CALCIUM UPTAKE

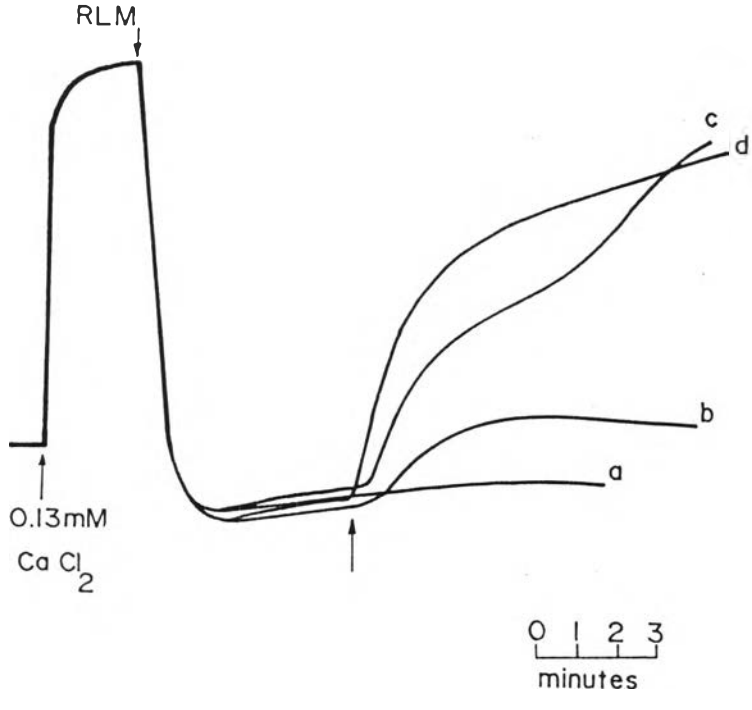


trol

μM bezafibrate } added initially

μM bezafibrate }

CALCIUM RELEASE



a - control

b - 50 μM bezafibrate } added 5 min af

c - 100 μM bezafibrate } RLM

d - 0.05 mM DNP }

Figure 21. Effects of bezafibrate and ruthenium red on calcium transport by rat liver mitochondria with glutamate plus malate as substrates.

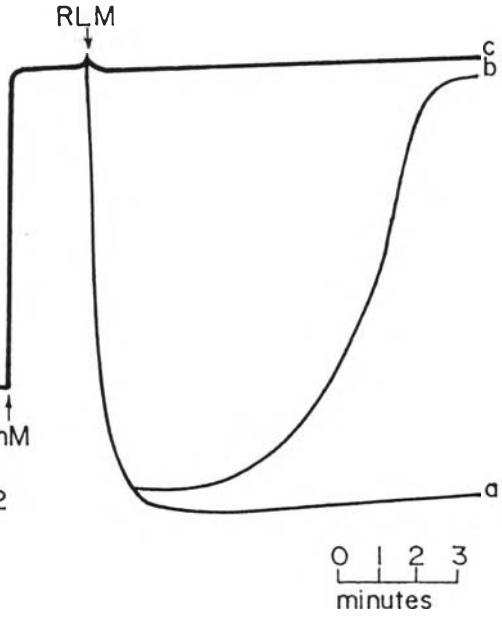
Left panel: Effect on mitochondrial calcium uptake. Composition of reaction system: 37.33 mM HEPES buffer pH 7.2, 1.87 mM $MgCl_2$, 85.87 mM KCl, 0.17 mM potassium phosphate, 14.17 mM sucrose, 5.0 mM potassium glutamate, 5.0 mM potassium malate, and 0.13 mM $CaCl_2$. Absolute ethanol, bezafibrate, and ruthenium red were added initially. The mitochondrial protein was 1.74 mg/ml. Total volume 3.0 ml.

Right panel: Effect on mitochondrial calcium release. Composition of reaction system: 37.33 mM HEPES buffer pH 7.2, 1.87 mM $MgCl_2$, 85.87 mM KCl, 0.17 mM potassium phosphate, 14.17 mM sucrose, 5.0 mM potassium glutamate, 5.0 mM potassium malate and 0.13 mM $CaCl_2$. Absolute ethanol, bezafibrate and ruthenium red were added 5 min after mitochondria. The mitochondrial protein was 1.74 mg/ml. Total volume 3.0 ml.

In both panels: $CaCl_2$ was first added to calibrate the calcium-selective electrode. The distance of the upward deflection following $CaCl_2$ addition denotes the concentration in the medium of added calcium ion, i.e. 0.13 mM. Calcium transport was initiated by adding the

mitochondria (RLM). The upward and downward deflections indicate the increase and decrease of calcium ion concentration in the reaction mixtures respectively.

CALCIUM UPTAKE

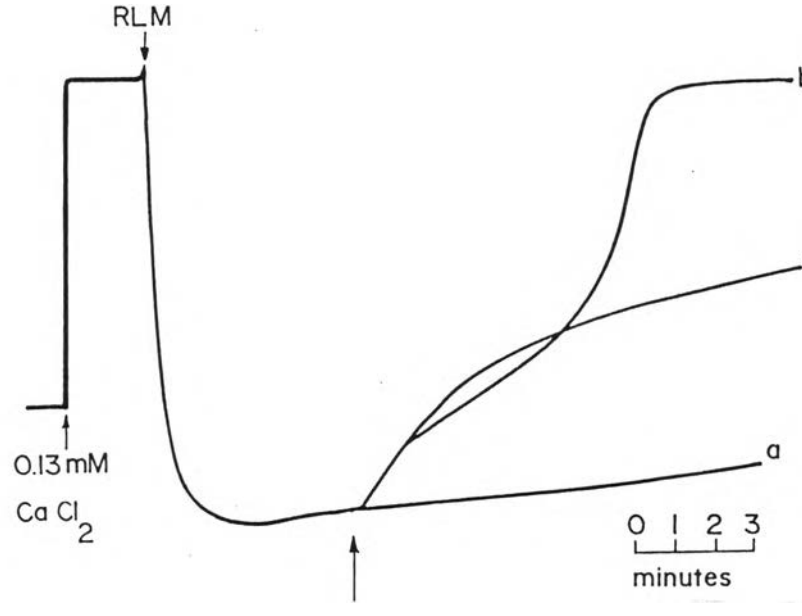


trol

μM bezafibrate } added initially

g ruthenium red

CALCIUM RELEASE



a - control

b - 100 μM bezafibrate } added 5 min afte

c - 3 μg ruthenium red } RLM

Table 8. Effect of bezafibrate on ATPase activity of rat liver mitochondria in the presence and absence of DNP.

Experiments	ATPase activity (μmole Pi/10 min/mg protein)
No DNP	
Control	0.33±0.03
50 μM bezafibrate	0.34±0.03
100 μM bezafibrate	0.38±0.02[a]
100 μM bezafibrate + 10 μg oligomycin	0.44±0.04
With 0.1 mM DNP	
Control	2.53±0.07
50 μM bezafibrate	2.36±0.10[b]
100 μM bezafibrate	2.32±0.09[b]
10 μg oligomycin	0.54±0.04[c]

a - $p < 0.05$ compared with control (no DNP)

b - $p < 0.05$ } compared with control

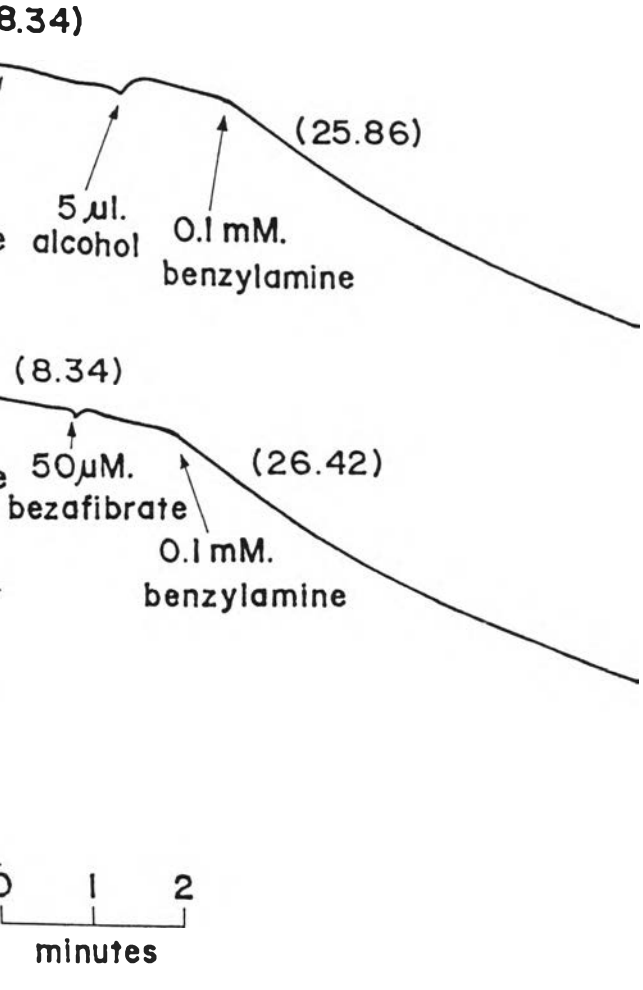
c - $p < 0.005$ } (with 0.1 mM DNP)

Composition of reaction system: 35.30 mM HEPES buffer pH 7.2,1.77 mM $MgCl_2$,81.19 mM KCl, 16.78 mM sucrose, 5.03 mM ATP, bezafibrate, DNP, and oligomycin as indicated.

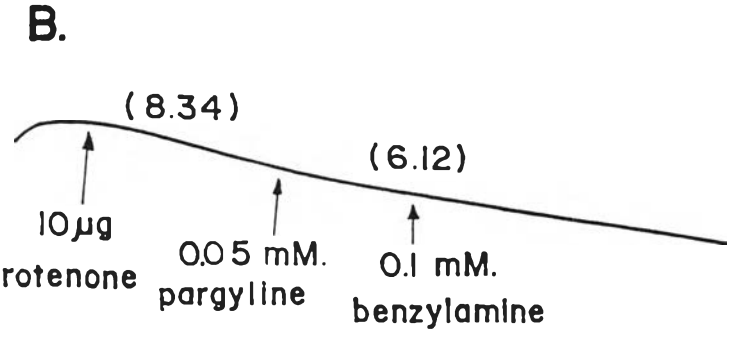
The average mitochondrial protein for "no DNP" and "with 0.1 mM DNP" experiments were 2.36 and 2.28 mg/ml respectively. Total volume 2.98 ml. Temperature 37°C. In the "no DNP" experiments, the mitochondria were preincubated with bezafibrate for 1 min before ATP was added. In the "with 0.1 mM DNP" experiments, bezafibrate was added 1 min after DNP and ATP added 1 min after bezafibrate. The reaction mixtures were further incubated for 10 min after ATP addition. Values are means±SEM from four experiments.

Figure 22. Effect of bezafibrate on monoamine oxidase (MAO) activity of rat liver mitochondria.

Composition of reaction system: 23.56 mM potassium phosphate pH 7.2, 13.09 mM sucrose, 10 µg rotenone, 0.1 mM benzylamine, bezafibrate and pargyline as indicated. The mitochondrial protein was 1.78 mg/ml. Total volume 1.91 ml. Temperature 37°C. The figures in parentheses are rates of oxygen consumption in ng-atoms O/ml/min.


8.34)
(25.86)
5 µl.
alcohol
0.1 mM.
benzylamine
(8.34)
50µM.
bezafibrate
(26.42)
0.1 mM.
benzylamine

0
1
2
minutes


B.
(8.34)
(6.12)
10µg
rotenone
0.05 mM.
pargyline
0.1 mM.
benzylamine

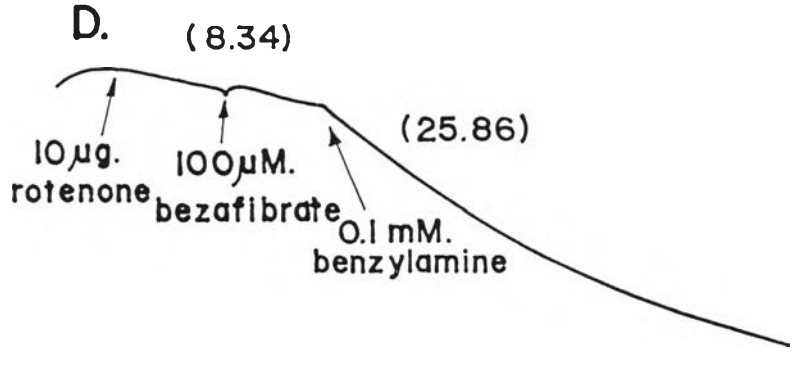

D.
(8.34)
10µg.
rotenone
100µM.
bezafibrate
(25.86)
0.1 mM.
benzylamine

Figure 23. Tracings comparing the effects of bezafibrate with clofibric acid on state 4, state 3, and state 3u respiration of rat liver mitochondria.

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate 5.24 mM potassium malate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, bezafibrate and clofibric acid as indicated. The mitochondrial protein was 2.48 mg/ml. Total volume 1.91 ml. Temperature 37^{o}C. The figures in parentheses are rates of oxygen consumption in ng-atoms O/ml/min.

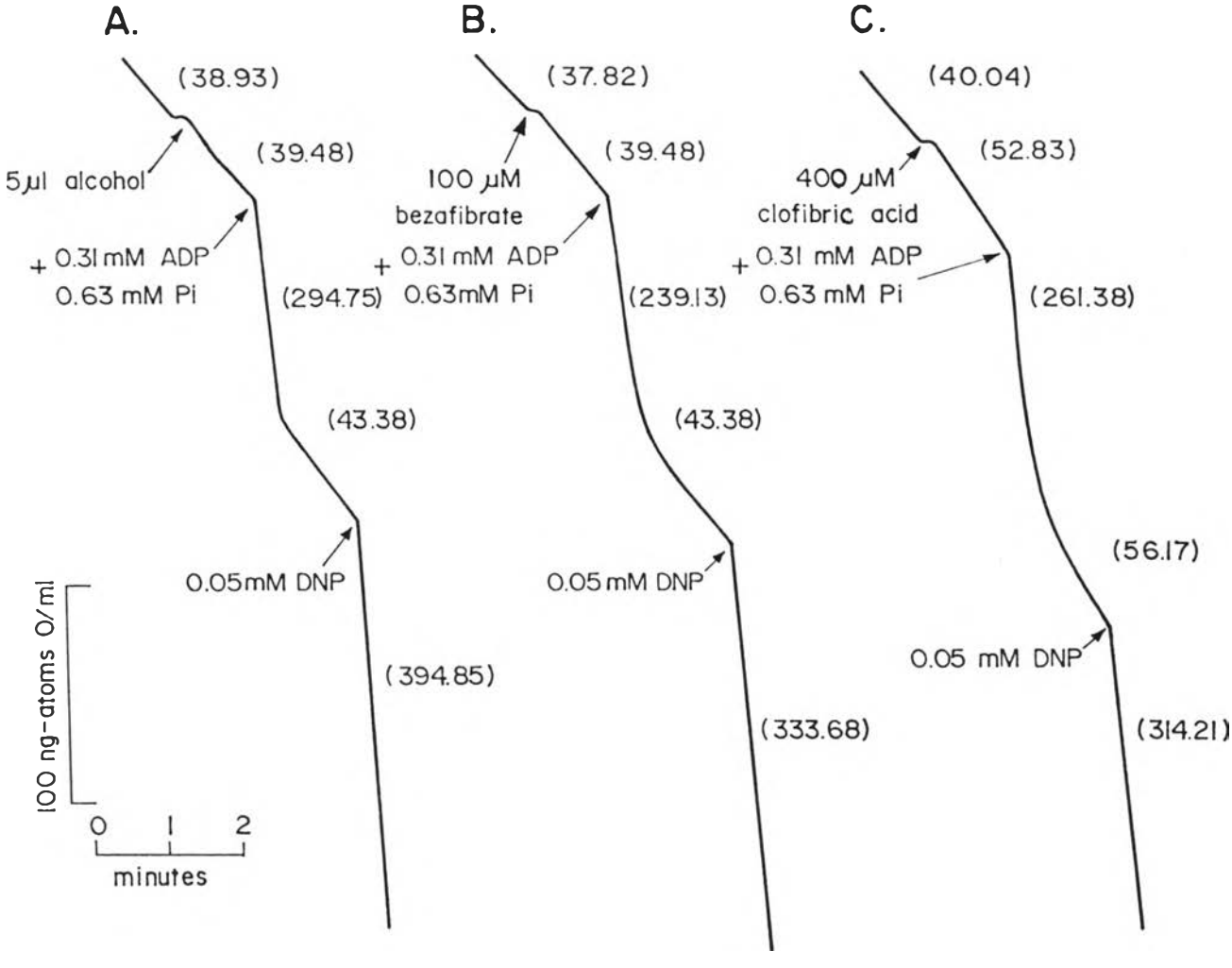

A.
(38.93)
5µl alcohol
(39.48)
+ 0.31 mM ADP
0.63 mM Pi
(294.75)
(43.38)
0.05mM DNP
(394.85)
100 ng-atoms O/ml
0
1
2
minutes
B.
(37.82)
100 µM
bezafibrate
(39.48)
+ 0.31 mM ADP
0.63mM Pi
(239.13)
(43.38)
0.05 mM DNP
(333.68)
C.
(40.04)
400 µM
clofibric acid
(52.83)
+ 0.31 mM ADP
0.63 mM Pi
(261.38)
(56.17)
0.05 mM DNP
(314.21)

Table 9. Effects of bezafibrate compared with clofibric acid on state 4, state 3 and state 3u respiration of rat liver mitochondria.

Experiments	Respiratory rates (ng-atoms O/min/mg protein)
	state 4
Control	17.65±0.60
100 μM bezafibrate	17.65±0.60
400 μM clofibric acid	22.88±0.56[a]
	state 3
Control	122.51±1.91
100 μM bezafibrate	97.11±0.77[a]
400 μM clofibric acid	105.17±0.37[a]
	state 3u
Control	158.62±1.11
100 μM bezafibrate	133.43+1.03[a]
400 μM clofibric acid	123.54±2.02[a,b]

a - $p < 0.005$ compared with control

b - $p < 0.05$ compared with 100 μM bezafibrate

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM

sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium malate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, bezafibrate and clofibric acid as indicated. The average mitochondrial protein was 1.96 mg/ml. Total volume 1.91 ml. Temperature 37^{o}C. ADP+Pi were added 1 min after bezafibrate or clofibric acid and DNP added during state 4 respiration. Values are means±SEM from four experiments.

Table 10. Effects of bezafibrate compared with clofibric acid on respiratory control index (RCI) and P/O ratio of rat liver mitochondria.

Experiments	RCI	P/O
Control	6.11+0.23	2.72±0.13
100 μM bezafibrate	4.77±0.25[a]	2.71±0.07
400 μM clofibric acid	4.38±0.10[a]	2.53±0.06

a - $p < 0.005$ compared with control

Composition of reaction system: 37.70 mM HEPES buffer pH 7.2, 1.88 mM $MgCl_2$, 86.70 mM KCl, 13.09 mM sucrose, 5.24 mM potassium glutamate, 5.24 mM potassium matate, 0.31 mM ADP + 0.63 mM Pi, 0.05 mM DNP, bezafibrate, and clofibric acid as indicated. The average mitochondrial protein was 1.96 mg/ml. Total volume 1.91 ml. Temperature 37°C. The mitochondria were preincubated with bezafibrate or clofibric acid for 1 min before ADP + Pi were added. The RCI values and P/O ratios are expressed in means±SEM from four experiments.

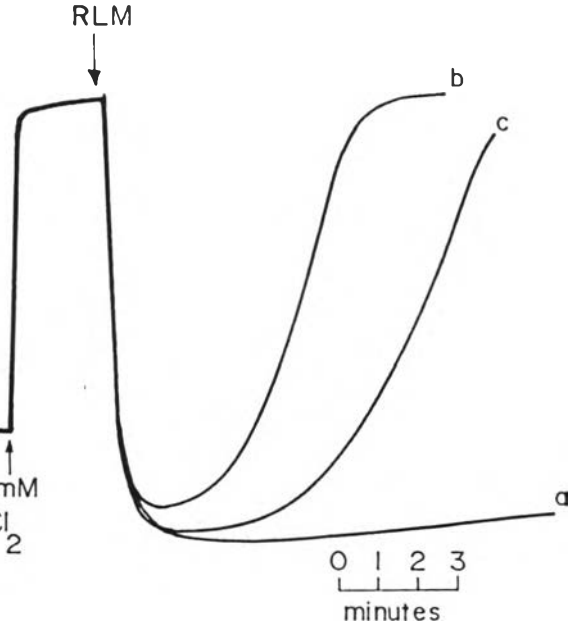
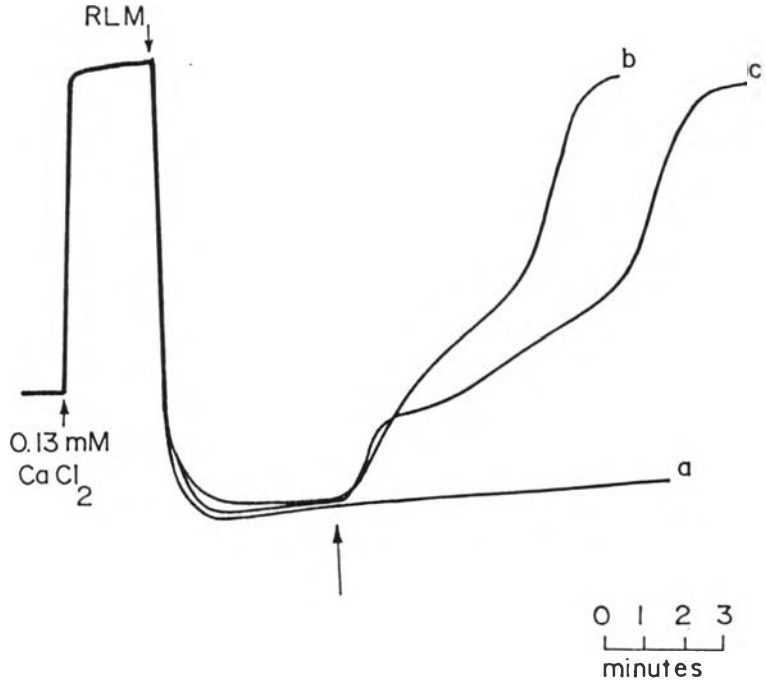
Figure 24. Comparison of the effects of bezafibrate and clofibric acid on calcium transport by rat liver mitochondria with glutamate plus malate as substrates.

Left panel : Effect on mitochondrial calcium uptake. Composition of reaction system: 37.46 mM HEPES buffer pH 7.2, 1.87 mM $MgCl_2$, 86.15 mM KCl, 0.17 mM potassium phosphate, 12.54 mM sucrose, 5.02 mM potassium glutamate, 5.02 mM potassium malate, and 0.13 mM $CaCl_2$. Absolute ethanol, bezafibrate, and clofibric acid were added initially. The mitochondrial protein was 1.69 mg/ml. Total volume 2.99 ml.

Right panel :Effect on mitochondrial calcium release. Composition of reaction system: 37.46 mM HEPES buffer pH 7.2, 1.87 mM $MgCl_2$, 86.15 mM KCl, 0.17 mM potassium phosphate, 12.54 mM sucrose, 5.02 mM potassium glutamate, 5.02 mM potassium malate, and 0.13 mM $CaCl_2$. Absolute ethanol, bezafibrate, and clofibric acid were added 5 min after mitochondria . The mitochondrial protein was 1.69 mg/ml. Total volume 2.99 ml.

In both panels: $CaCl_2$ was first added to calibrate the calcium-selective electrode. The distance of the upward deflection following $CaCl_2$ addition denotes the concentration in the medium of added calcium ion, i.e.

0.13 mM. Calcium transport was initiated by adding the mitochondria (RLM). The upward and downward deflections indicate the increase and decrease of calcium ion concentration in the reaction mixtures respectively.

CALCIUM UPTAKE	CALCIUM RELEASE
	
rol μM bezafibrate } added initially μM clofibric acid	a - control b - 100 μM bezafibrate } added 5 min afte c - 400 μM clofibric acid } RLM