CHAPTER III

Results

Effects of ketoconazole on oxidative phosphorylation by isolated rat liver mitochondria

Typical tracings demonstrating the effect of ketoconazole on mitochondrial oxygen consumption with glutamate plus malate as substrate are shown in figure 19. Curve A is the control response of the mitochondria to the addition 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 3u respiration. This respiratory stimulation proceeded until all the dissolved oxygen in the incubation medium was consumed. The RCI calculated from curve A was 6.23 indicating that the mitochondria were tightly coupled. Ketoconazole at 20 μg/ml drastically depressed both states 3 and 3u respiration and reduced the RCI to 1.37 (curve B); increasing the dose to 40 μg/ml produced stronger inhibition (curve C). The inhibitory effect of ketoconazole on states 3 and 3u respiration was dose-dependent as shown in

figure 20; and the inhibition appeared to level off when the doses exceeded 40 μg/ml.

It is well known that glutamate plus malate are NAD^+-linked substrates which donate electrons to mitochondrial respiratory chain via complex I. Further experiments were performed with succinate which donates electron to the respiratory chain via complex II, as substrate. As can be seen from figure 21, ketoconazole from 10-100 μg/ml had slight inhibitory and stimulatory effects on states 3 and 3u respiration respectively. In both cases, the effects were apparently not dose related. This result indicated that ketoconazole probably inhibited oxidative phosphorylation by blocking electrons flow at complex I in the mitochondrial respiratory chain. This notion was supported by experiments in which other NAD^+-linked substrates, i.e., α-ketoglutarate(figure 22), β-hydroxybutyrate(figure 23) and pyruvate plus malate(figure 24) were used. With these substrates, ketoconazole was found to produce a dose-dependent inhibition of states 3 and 3u respiration similar to that observes with glutamate plus malate.

Effect of ketoconazole on mitochondrial oxidation of NADH.

The effect of ketoconazole on NADH oxidation by osmotic-shocked rat liver mitochondria is recorded in figure 25. In these experiments, the mitochondria were prior

treated with hypotonic sucrose solution to increase the inner membrane permeability to exogenous NADH. Addition of 1μmole NADH to osmotic-shocked mitochondria stimulated respiration as NADH was oxidized by the respiratory chain(curve A). The NADH-stimulated respiration proceeded until the dissolved oxygen in the reaction mixture was exhausted. Ketoconazole at 20 μg/ml clearly depressed the respiratory stimulation evoked by NADH(curve B). This result strongly indicated that ketoconazole had inhibitory effect on mitochondrial respiratory chain and agreed with the effect of ketoconazole on states 3 and 3u respiration by intact mitochondria respiring with various NAD^+-linked substrates reported above.

Factors influencing the effect of ketoconazole on oxidative phosphorylation by isolated rat liver mitochondria.

1. Effect of pH

The influence of pH on the inhibitory action of ketoconazole on state 3 and 3u respiration with glutamate plus malate as substrates is recorded in table 3. It is seen that the percent inhibition of states 3 and 3u respiration produced by 20 μg/ml ketoconazole increased from 69.79 to 74.45% and from 73.61 to 75.42% when the medium pH was raised from 6.8 to 7.6 respectively. Thus an increase of

pH by 0.8 unit only slightly increased the depressive action of ketoconazole on mitochondrial oxidative phosphorylation.

2. Effect of dithiothreitol (DTT).

Dithiothreitol(DTT), a sulfhydryl-protecting substance, was used in this experiment to investigate if ketoconazole acts by combining with mitochondrial sulfhydryl group. As reported in figures 26 and 27. DTT, 1.05 mM, did not reverse the inhibitory effect of ketoconazole 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 bovine serum albumin (BSA)

In the introduction chapter, ketoconazole can bind to serum albumin. It is therefore interesting to study if BSA can attenuate effect of ketoconazole by binding and thereby removing the drug from the mitochondria. In this experiment, the study on state 3u respiration was omitted because BSA forms complex with DNP and inhibits the uncoupling effect. As shown in figure 28, 20 mg BSA had little effect on state 3 respiration whereas 20 μg/ml ketoconazole decreased rate from 165.82±2.45 to 35.67±4.32 ng-atoms O/min/mg protein. Addition of 5,10

or 20 mg BSA clearly antagonized the inhibitory effect of ketoconazole on state 3 respiration.

Effect of ketoconazole on calcium-stimulated respiration by isolated rat liver mitochondria.

When calcium is added to respiring mitochondria, it is accumulated by the mitochondria and simultaneously stimulates mitochondrial oxygen consumption. The effect of ketoconazole on calcium-stimulated respiration with glutamate plus malate as substrates is shown in figure 29. It is seen that ketoconazole produced a dose-dependent inhibition of calcium-stimulated respiration similar to that observed with states 3 and 3u respiration. Since calcium stimulates oxygen consumption when it is accumulated by mitochondria, this finding therefore indicated depressive action of ketoconazole on mitochondrial calcium transport.

Effect of ketoconazole on ATPase activity of isolated rat liver mitochondria.

The ATPase reaction is generally believed to represent the reversed process of the ATP synthase reaction. Agents which inhibit ATP synthesis, for example oligomycin, also depress the uncoupler-induced ATPase activity . The effect of ketoconazole on mitochondrial ATPase activity with and without DNP is shown in result table 4. In the absence of DNP, the

control ATPase activity was very low since the enzyme functions in the direction of ATP synthesis. Ketoconazole at 20 and 40 μg/ml produced no effect on the enzyme 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. Ketoconazole at 20 and 40 μg/ml also had no effect on the DNP-stimulated ATPase activity.

Effect of ketoconazole on mitochondrial monoamine oxidase (MAO) activity

The effect of ketoconazole on MAO activity by isolated rat liver mitochondria is shown in the figure 30. 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 addition of benzylamine. The initial rate of oxygen consumption was slow but when benzylamine was added the rate increased threefolds(from 8.35 to 25.88 ng-atoms O/ml/min). Pargyline, a MAO inhibitor, completely blocked the oxygen consumption induced by benzylamine (Curve B). Ketoconazole, at 20 μg/ml, completely blocked the benzylamine-induced

increase in oxygen uptake indicating that MAO activity was inhibited by the drug (curve C.).

THE FIGURES AND TABLES

Figure 19. Tracings demonstrating inhibitory effect of ketoconazole 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 20 μg/ml ketoconazole. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C.

* The figures in parentheses denote rate of oxygen consumption in ng-atoms/ml/min.

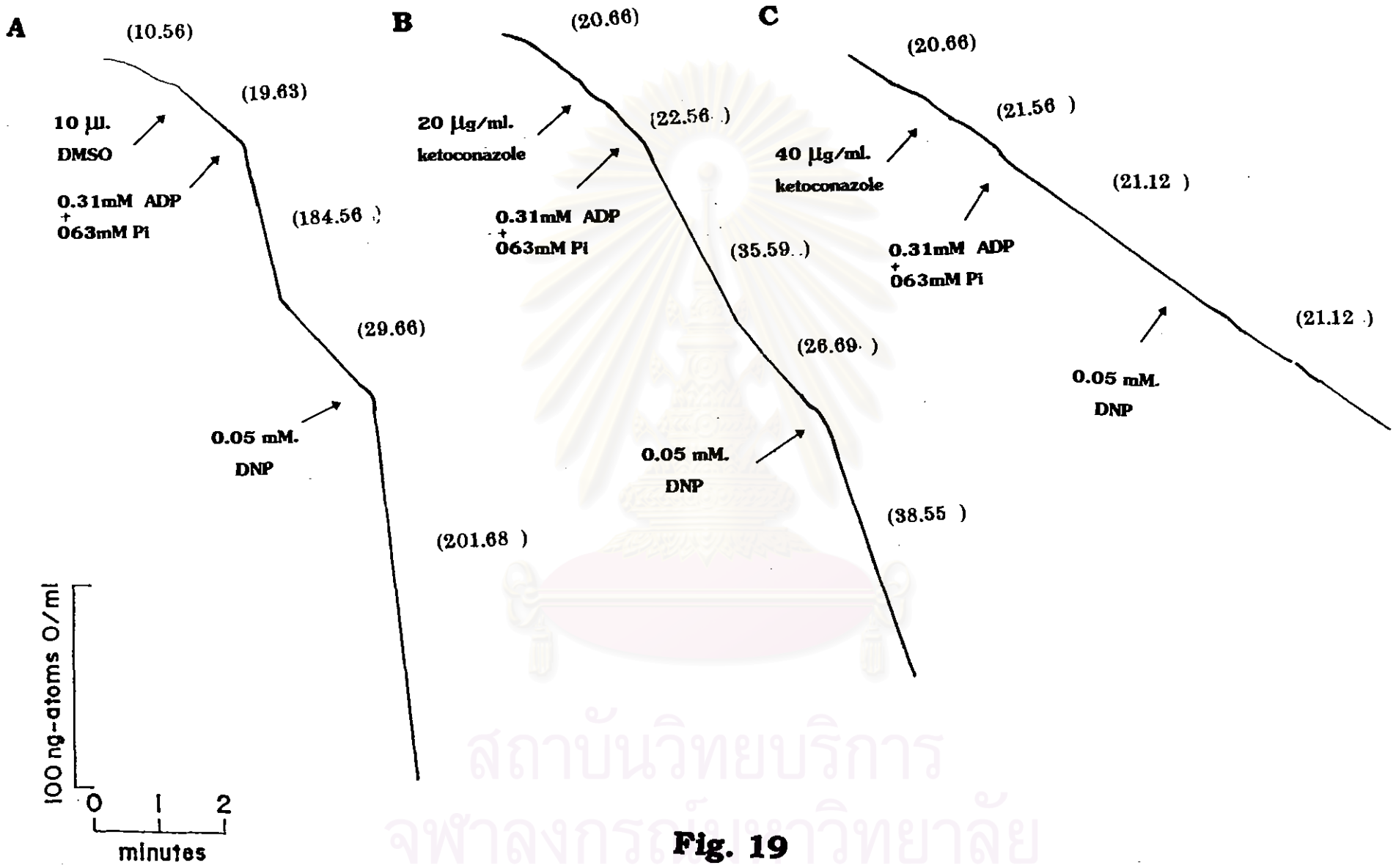


Fig. 19

Figure 20. Inhibition by ketoconazole of state 3 and state 3u respiration of rat liver mitochondria with glutamate plus malate 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 glutamate, 5.24 mM potassium malate, 0.31 mM ADP+ 0.63 mM Pi, 0.05 mM DNP and ketoconazole as indicated. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP+Pi were added 1 min after ketoconazole, DNP added during state 4 respiration. Each point represents a mean $\pm$ sem from four experiments.

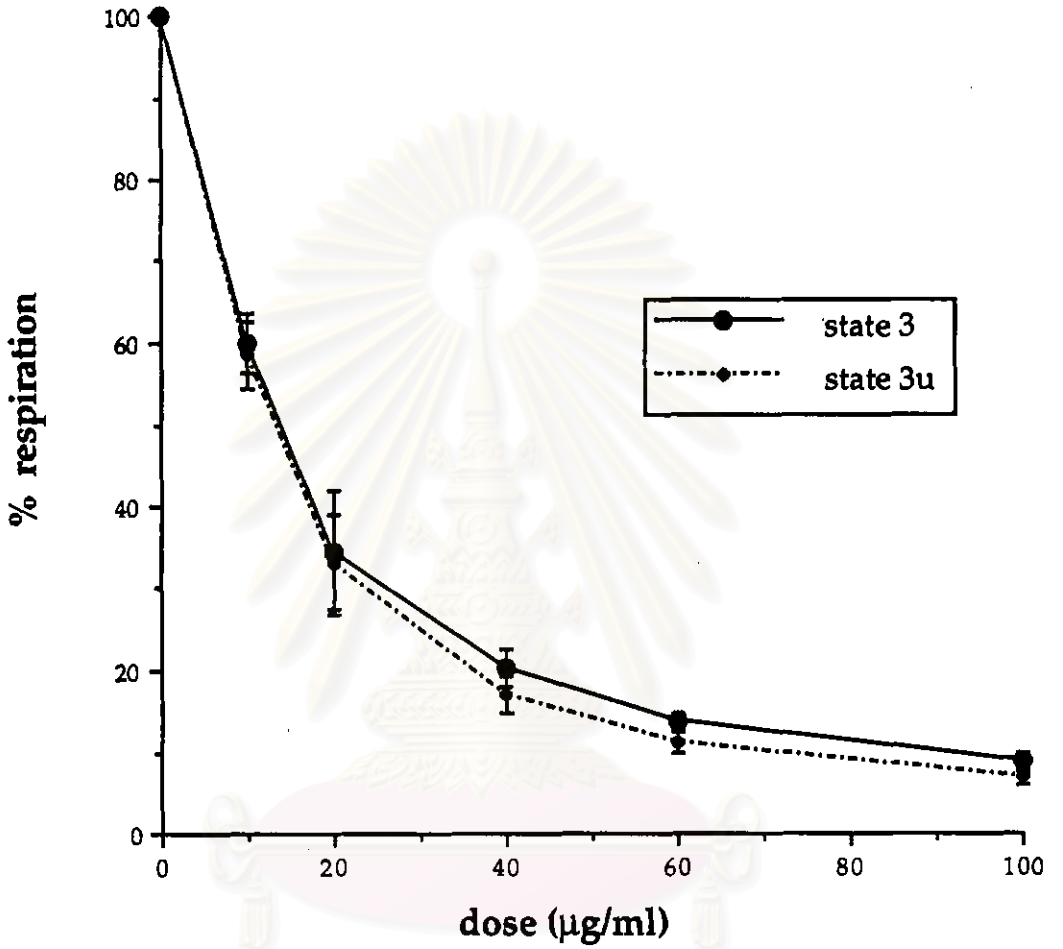


Fig. 20

Figure 21. Effect of ketoconazole on 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 ketoconazole as indicated. The average mitochondrial protein was 2.7 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP+Pi were added 1 min after ketoconazole ; DNP added during state 4 respiration. Each point represents a mean ± sem from four experiments.

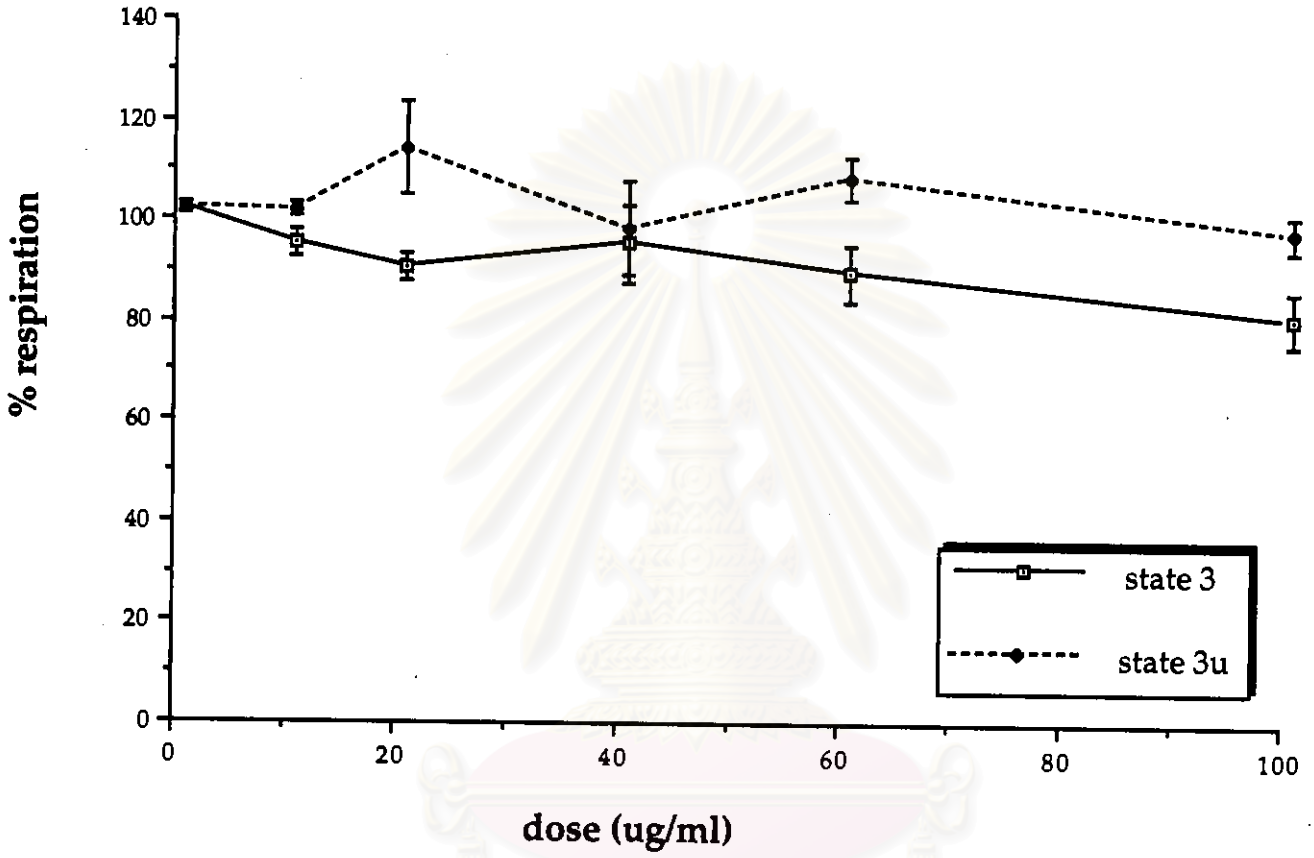


Fig. 21

Figure 22. Inhibition by ketoconazole of state 3 and state 3u respiration of rat liver mitochondria with α-ketoglutarate 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 α-ketoglutarate, 0.31 mM ADP+ 0.63 mM Pi, 0.05 mM DNP and ketoconazole as indicated. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP+Pi were added 1 min after ketoconazole; DNP added during state 4 respiration. Each point represents a mean ± sem from four experiments.

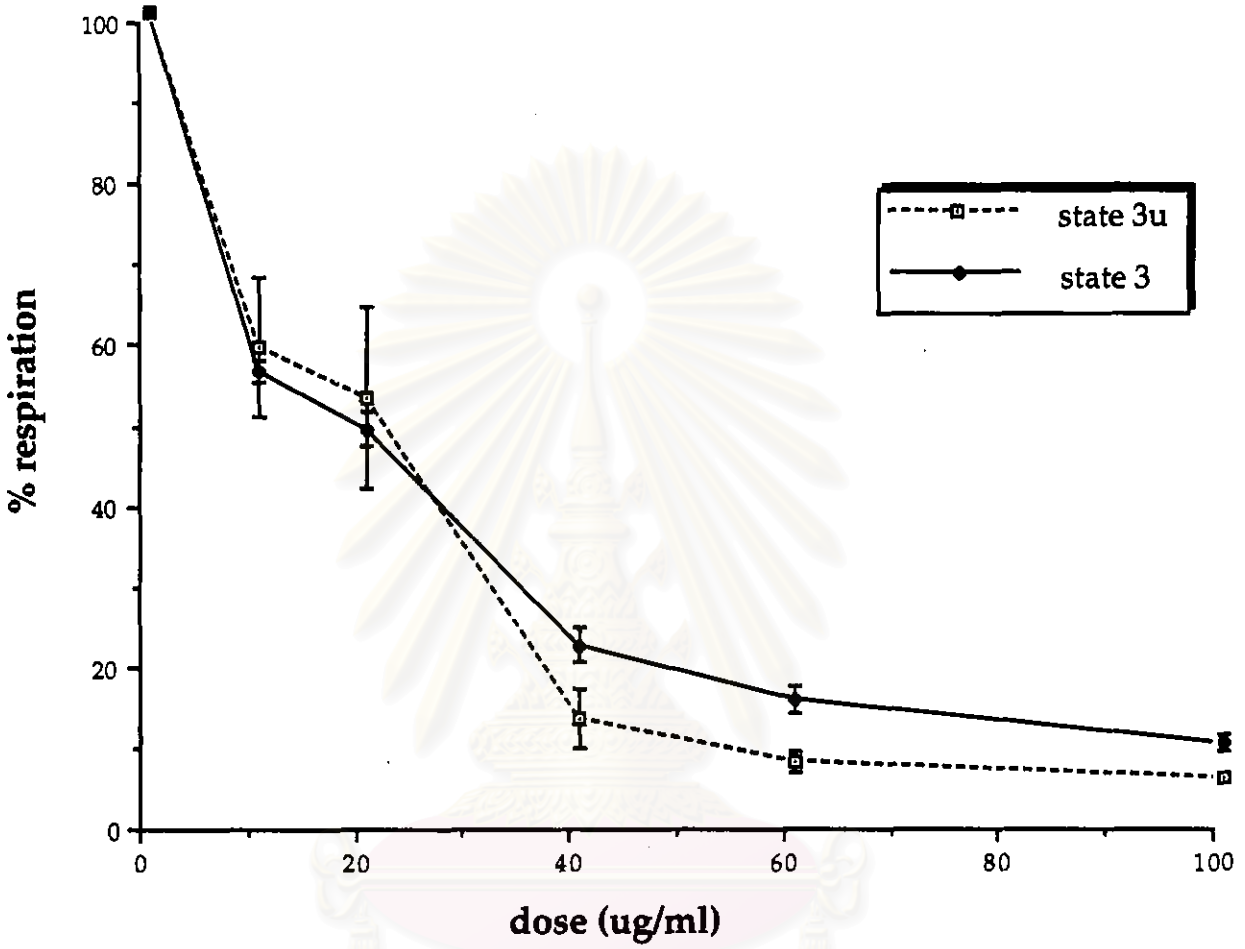


Fig. 22

Figure 23. Inhibition by ketoconazole of state 3 and state 3u respiration of rat liver mitochondria with β-hydroxybutyrate 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 β-hydroxybutyrate, 0.31 mM ADP+ 0.63 mM Pi, 0.05 mM DNP and ketoconazole as indicated. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP+Pi were added 1 min after ketoconazole; DNP added during state 4 respiration. Each point represents a mean ± sem from four experiments.

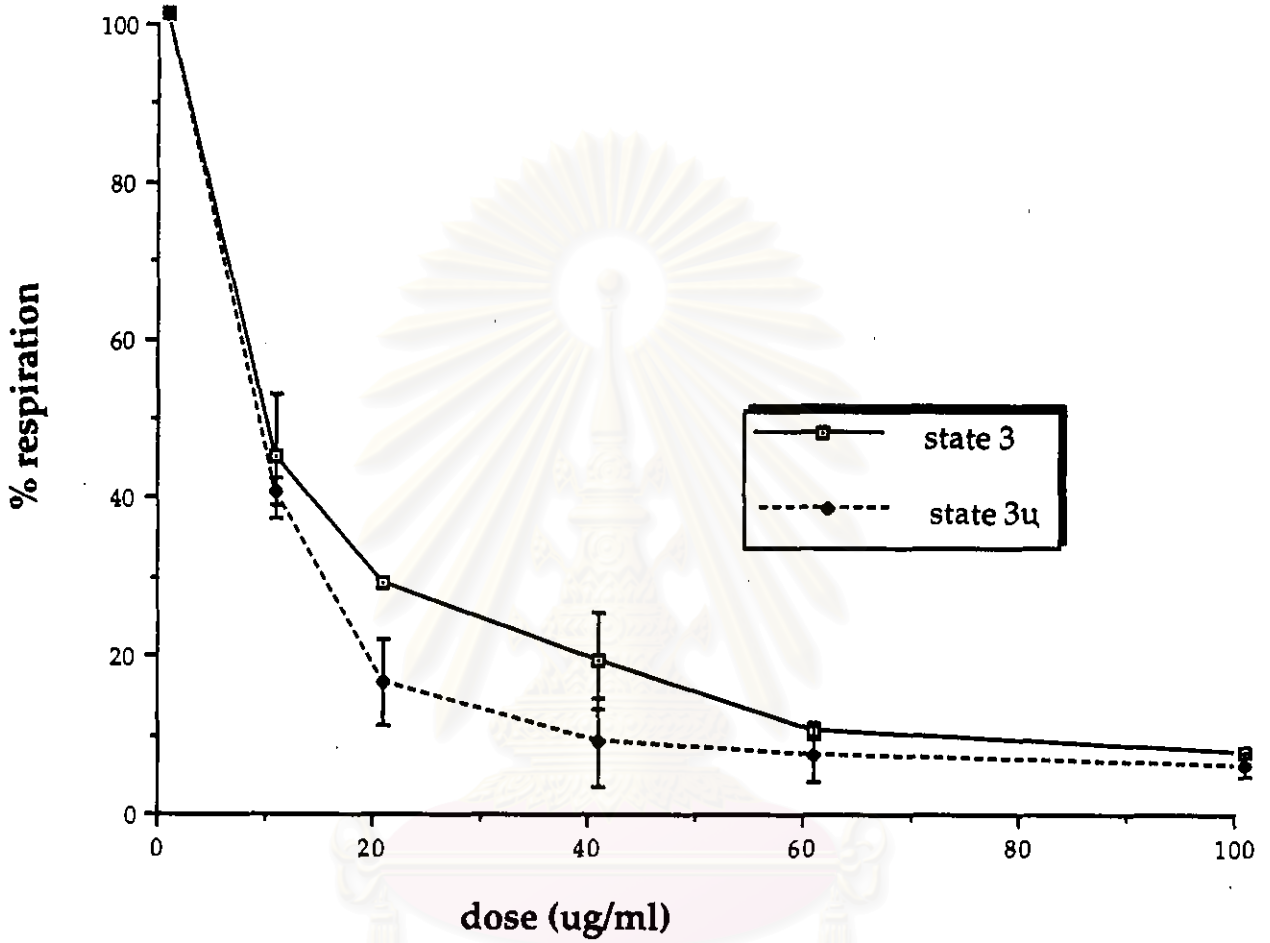


Fig. 23

Figure 24. Inhibition by ketoconazole of state 3 and state 3u respiration of rat liver mitochondria with pyruvate 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 pyruvic acid, 5.24 mM potassium malate, 0.31 mM ADP+ 0.63 mM Pi, 0.05 mM DNP and ketoconazole as indicated. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP+Pi were added 1 min after ketoconazole; DNP added during state 4 respiration. Each point represents a mean $\pm$ sem from four experiments.

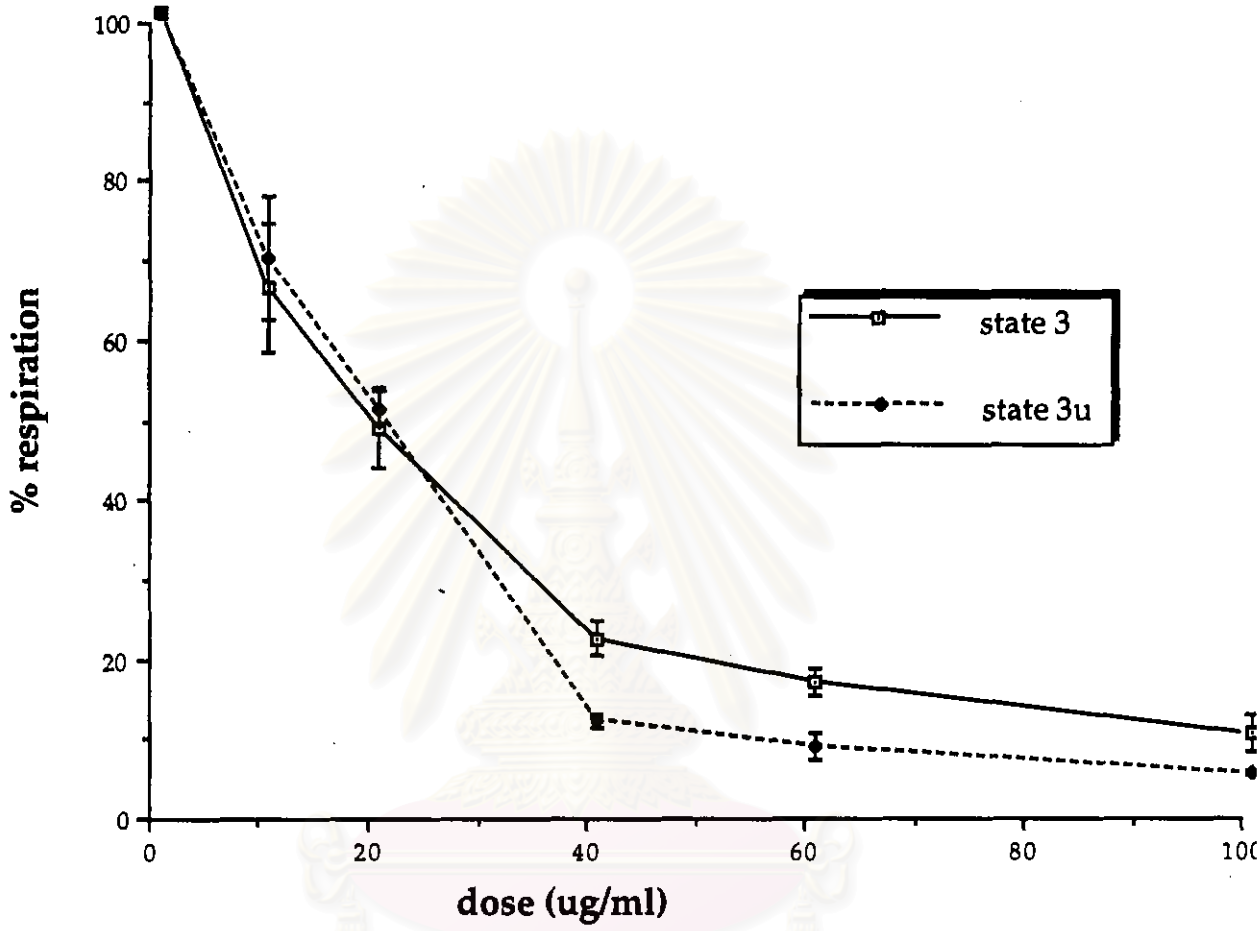


Fig. 24

Figure 25. Effect of ketoconazole on NADH-stimulated respiration of osmotic-shocked 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, 0.42 mM NADH and 20μg/ml ketoconazole. The mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C.

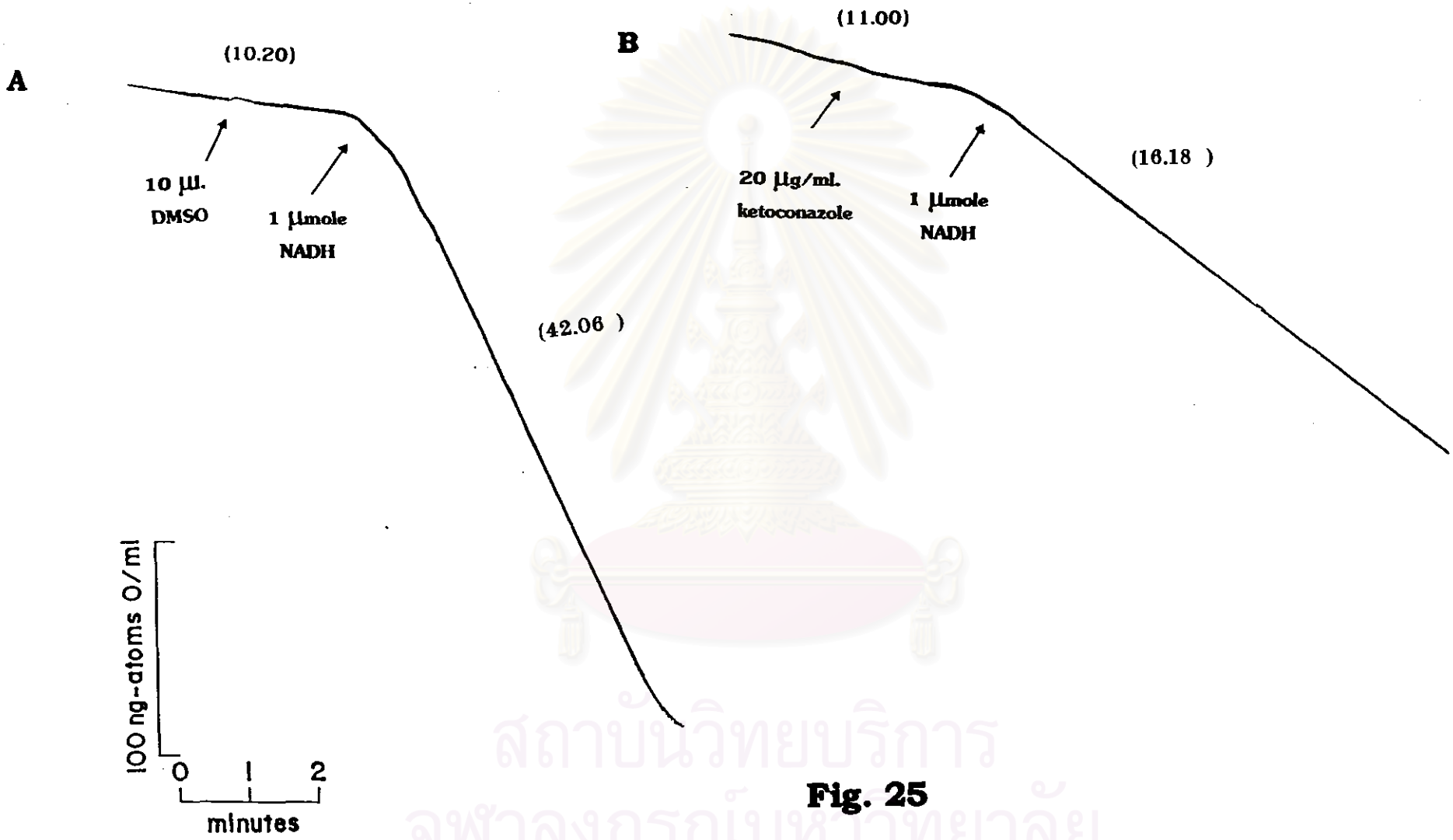


Fig. 25

Table 3. Influence of pH on inhibition by ketoconazole of state 3 and state 3u respiration of rat liver mitochondria with glutamate plus malate as substrates.

Experiments	rate of oxygen consumption (ng-atoms/min/mg protein)	
	state 3	state 3u
pH 6.8		
Control	113.89±4.33	128.13±4.00
+20 μg/ml ketoconazole	34.40±2.11	33.81±5.23
	(69.79%)	(73.61%)
pH 7.2		
Control	121.13±5.66	149.49±2.51
+20 μg/ml ketoconazole	32.33±2.90	37.89±2.85
	(73.53%)	(74.65%)
pH 7.6		
Control	99.66±3.89	102.34±1.27
+20 μg/ml ketoconazole	25.46±3.00	25.15±1.79
	(74.45%)	(75.43%)

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 20μg/ml ketoconazole . The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. ADP+Pi were added 1 min after ketoconazole. DNP added during state 4 respiration. Each value represents a mean ± sem from four experiments. The figures in parentheses denote % inhibition calculated from mean values of control and ketoconazole-treated experiments.

Figure 26. Illustrative tracings of the effect dithiothreitol (DTT) on the inhibitory effect of ketoconazole 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, 1.05 mM DTT and 20 μg/ml ketoconazole. The mitochondrial protein was 1.9 mg/ml. Total volume 1.91 ml. Temperature 37°C. The figures in parentheses denote rate of oxygen consumption in ng-atoms/ml/min.

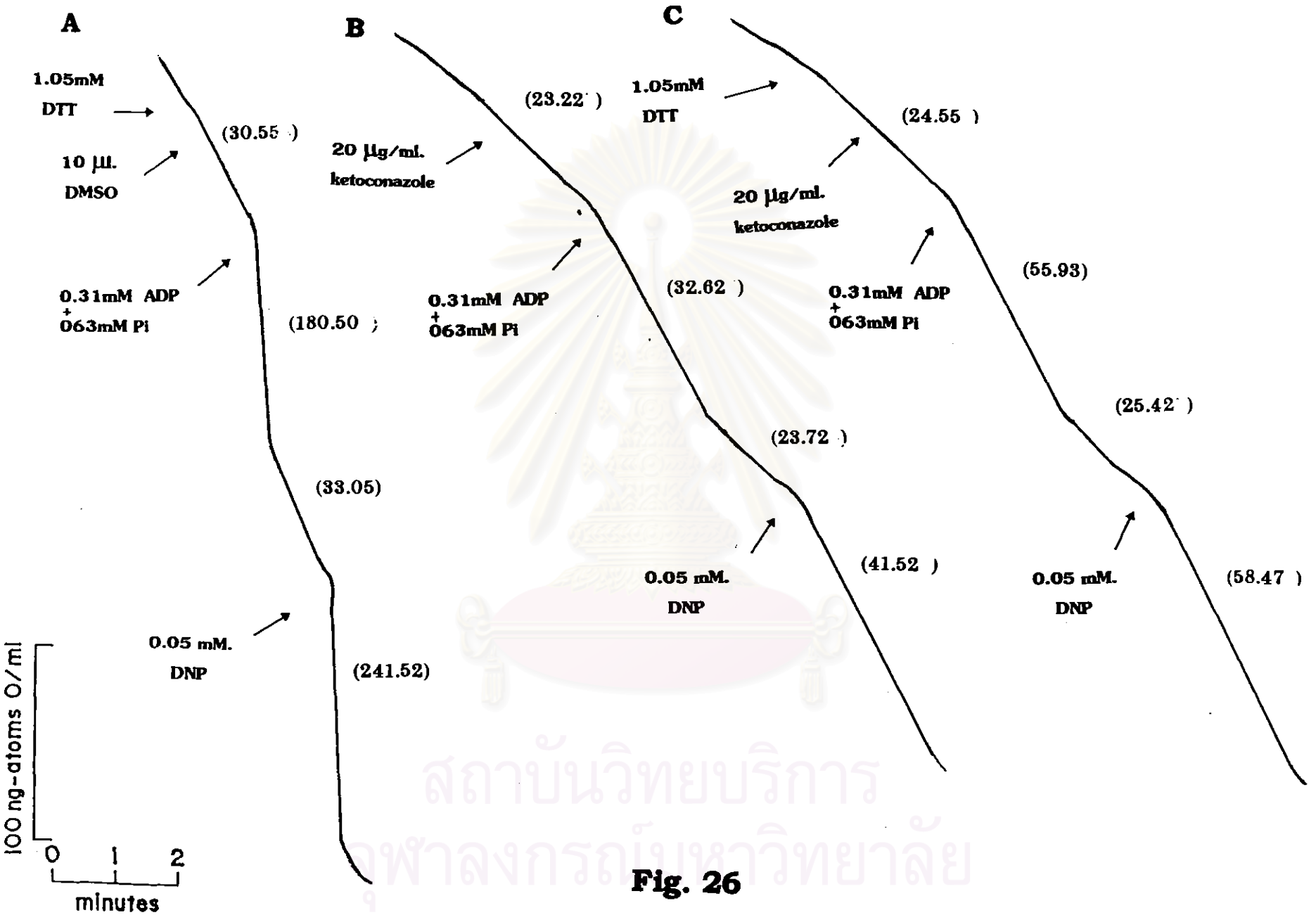
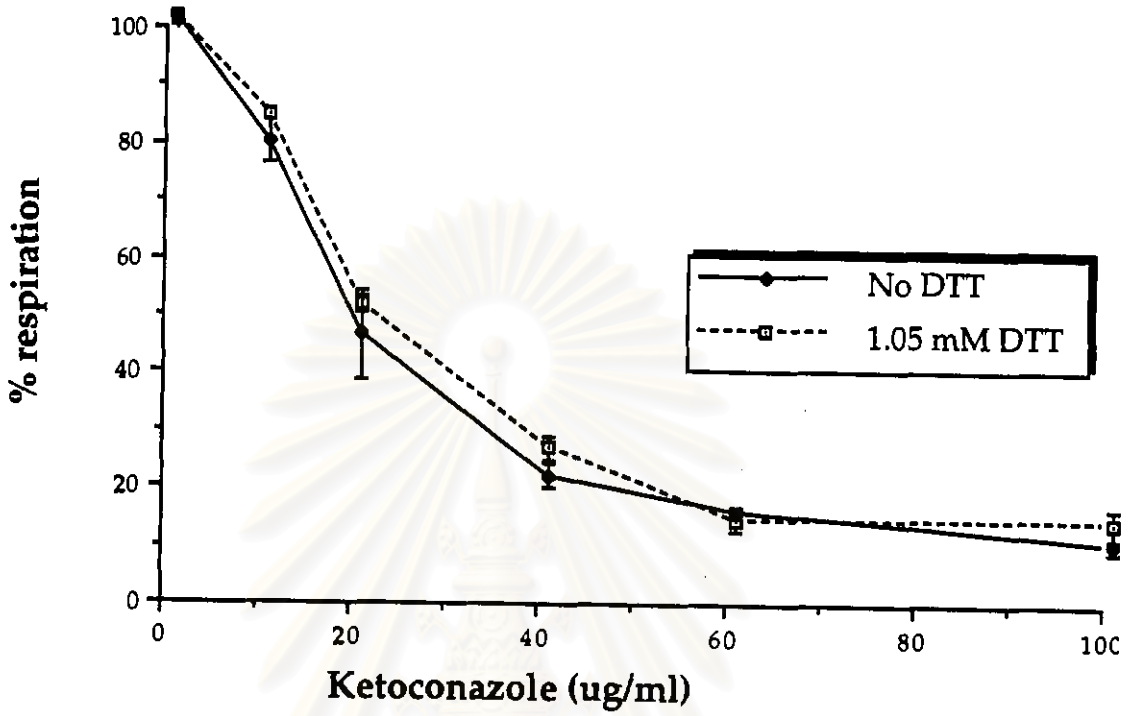


Fig. 26

Figure 27. Effect of dithiothreitol (DTT) on the inhibitory effect of ketoconazole 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, 1.05 mM DTT and 20 μg/ml ketoconazole. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. Ketoconazole was added 1 min after DTT. ADP+Pi were added 1 min after ketoconazole. DNP added during state 4 respiration. Each point represents a mean ± sem from four experiments.

STATE 3



STATE 3u

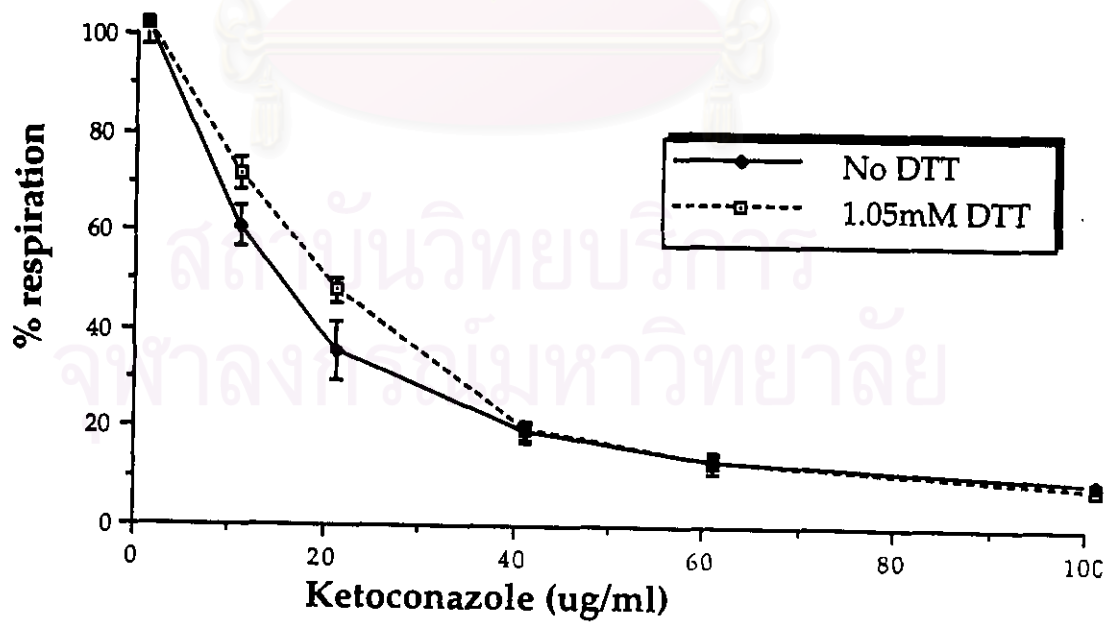


Fig. 27

Figure 28. Attenuation by bovine serum albumin (BSA) of ketoconazole induced inhibitory effect on state 3 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, 20 μg/ml ketoconazole, and BSA as indicated. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. Ketoconazole was added 1 min after BSA and ADP+Pi were added 1 min after ketoconazole. Each value represents a mean ± sem from four experiments.

* = $p<0.05$ compared with control (ketoconazole presented but no BSA added)

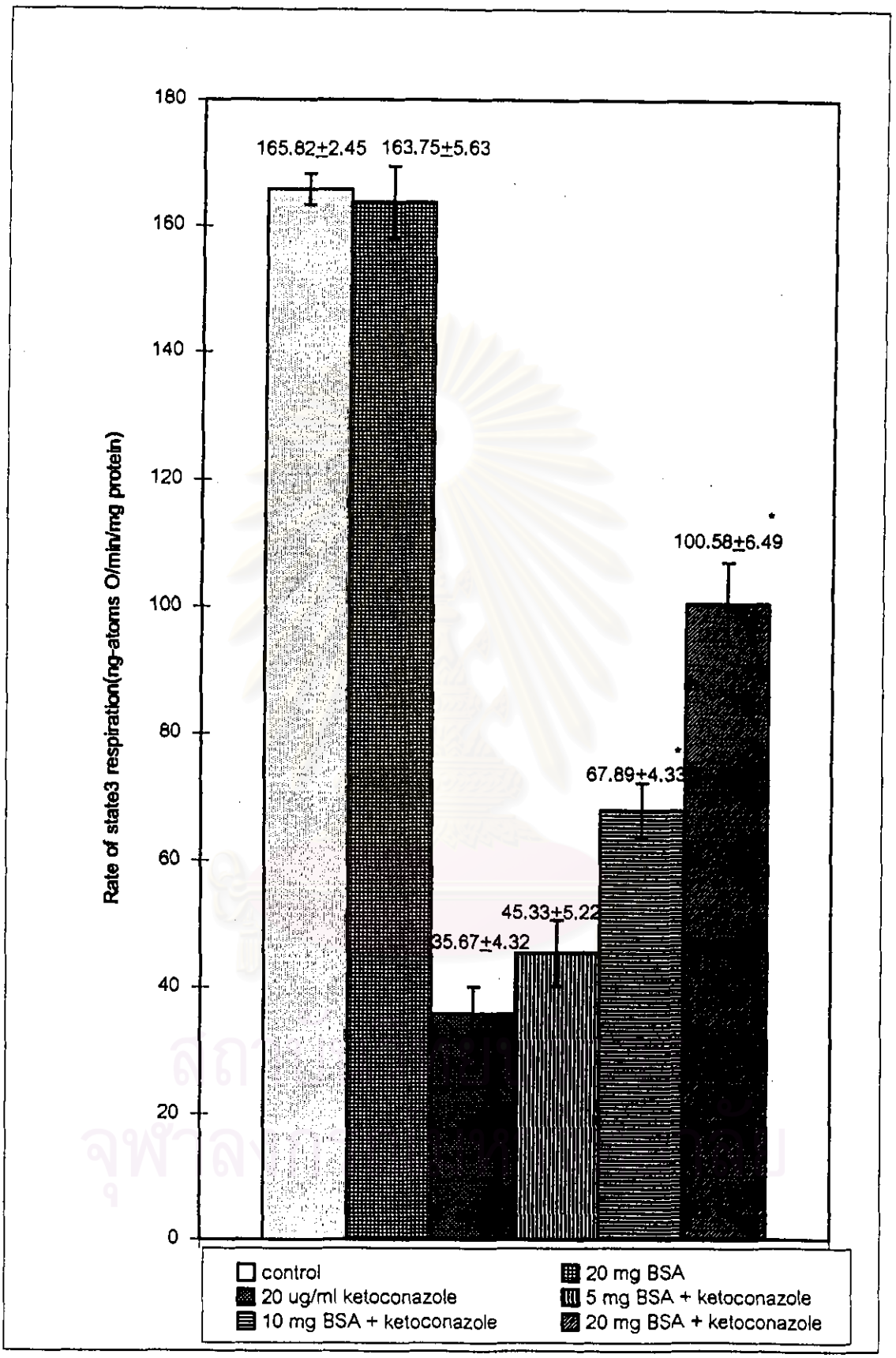

Rate of state3 respiration(ng-atoms O/min/mg protein)
180
160
140
120
100
80
60
40
20
0
165.82±2.45
163.75±5.63
35.67±4.32
45.33±5.22
67.89±4.33
100.58±6.49
control
20 ug/ml ketoconazole
10 mg BSA + ketoconazole
20 mg BSA
5 mg BSA + ketoconazole
20 mg BSA + ketoconazole

Fig. 28

Figure 29. Effect of ketoconazole on calcium-stimulated 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, 0.94 mM potassium phosphate, 5.24 mM potassium malate, 0.42 mM $CaCl_2$ and 20μg/ml ketoconazole. The average mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C. $CaCl_2$ were added 1 min after ketoconazole. Each value represents a mean ± sem from four experiments.

* = $p<0.05$ compared with control (no ketoconazole added)

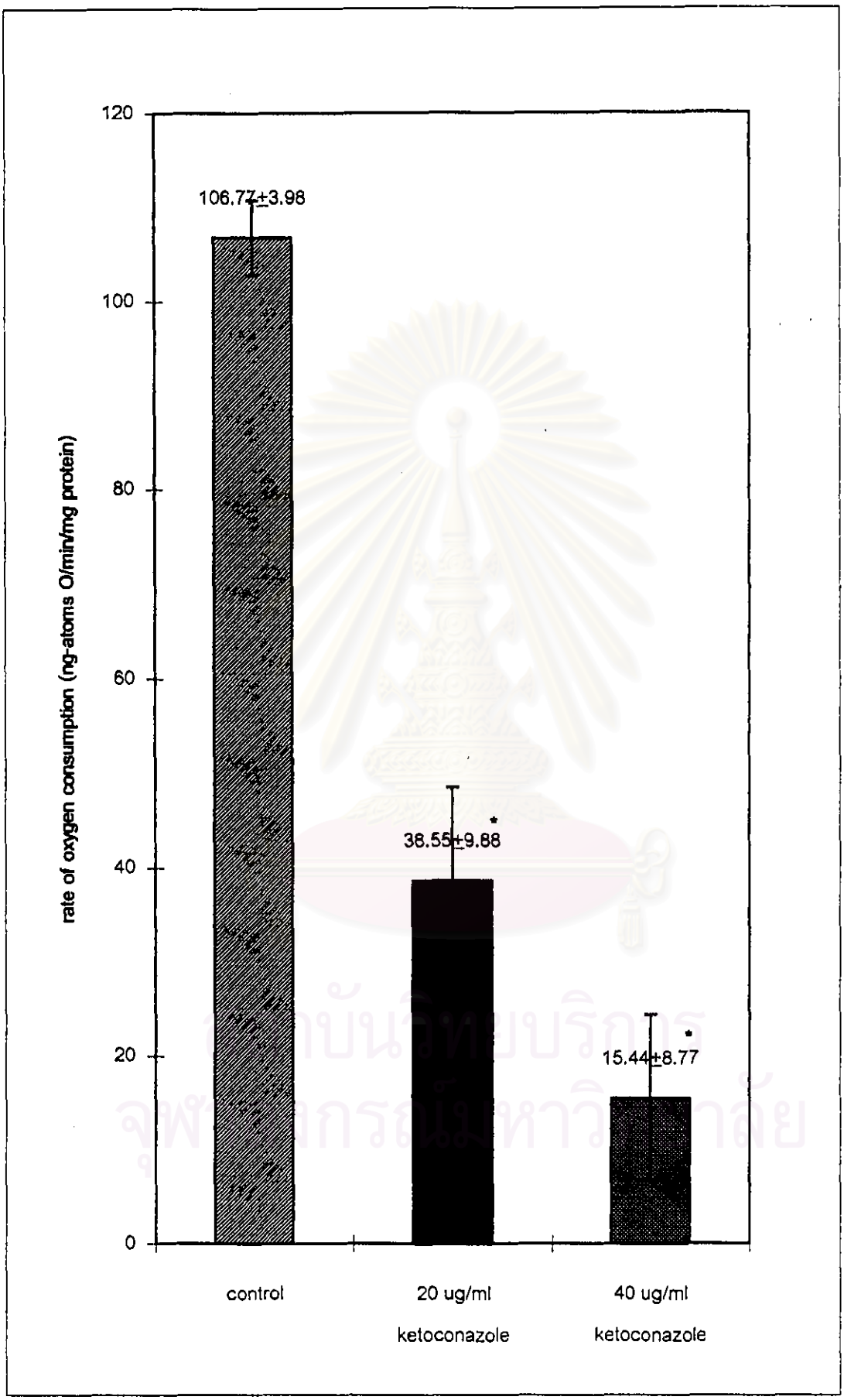

120
100
80
60
40
20
0
rate of oxygen consumption (ng-atoms O/min/mg protein)
106.77±3.98
38.55±9.88
*
15.44±8.77
*
control
20 ug/ml
ketoconazole
40 ug/ml
ketoconazole

Fig. 29

Table 4. Effect of ketoconazole on ATPase activity of rat liver mitochondria in the presence and absence of DNP.

Experiments	ATPase activity (μmole Pi/10min/mg protein)
No DNP	
Control	**0.34±0.05**
20μg/ml ketocoonazole	**0.32±0.06**
40μg/ml ketoconazole	**0.33±0.03**
With 0.1 mM DNP	
Control	**2.68±0.15**
10μg oligomycin	**0.40±0.15**
20μg/ml ketoconazole	**2.65±0.07**
40μg/ml ketoconazole	**2.67±0.10**

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, 5.03 mM ATP, 20μg/ml ketoconazole, 0.1 mM DNP and 10 μg oligomycin. The average mitochondrial protein for "with DNP" and "without DNP" experiments were 2.35 and 2.7 mg/ml respectively. Total volume 2.98 ml. Temperature 37°C. In the "without DNP" experiment, the mitochondria were preincubated with ketoconazole for 1 min before ATP was added. In the "with DNP" experiment, ketoconazole was added 1 min after DNP and ATP added 1

min after ketoconazole. The reaction mixtures were further incubated for 10 min after ATP addition. Each value represents a mean ± sem from four experiments.

Figure 30. Effect of ketoconazole 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, 0.05 mM pargyline and 20μg/ml ketoconazole. The mitochondrial protein was 1.8 mg/ml. Total volume 1.91 ml. Temperature 37°C.

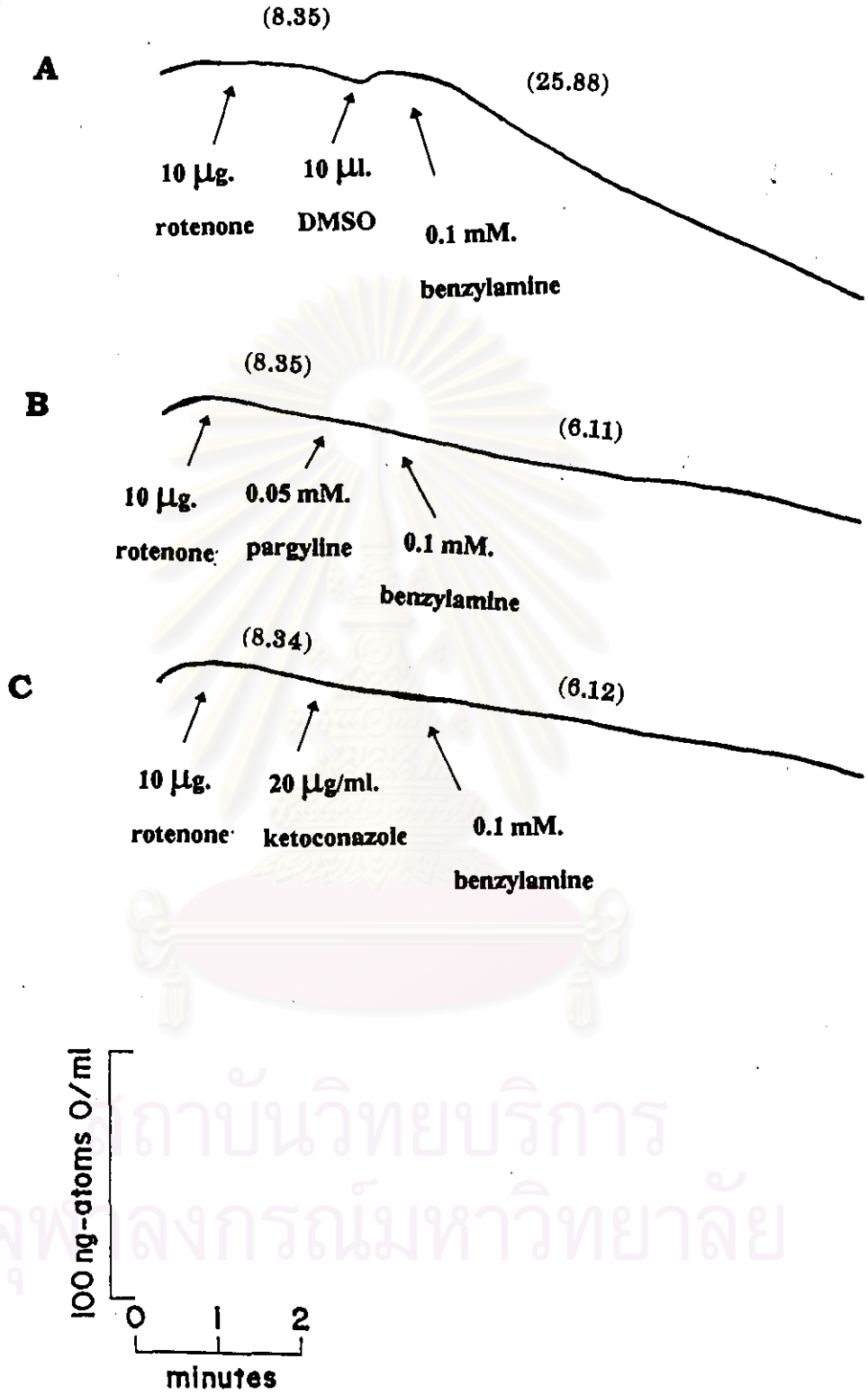

A
(8.35)
(25.88)
10 µg.
rotenone
10 µl.
DMSO
0.1 mM.
benzylamine
B
(8.35)
(6.11)
10 µg.
rotenone
0.05 mM.
pargyline
0.1 mM.
benzylamine
C
(8.34)
(6.12)
10 µg.
rotenone
20 µg/ml.
ketoconazole
0.1 mM.
benzylamine
100 ng-atoms O/ml
0
1
2
minutes

Fig. 30