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INHIBITION OF MITOCHONDRIAL OXIDATIVE METABOLISM BY SKF-525A IN INTACT CELLS AND ISOLATED MITOCHONDRIA

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Abstract—We have examined the effects of various concentrations of SKF-525A (β-diethylaminoethyldiphenylpropyl acetate · HCl) on the energy metabolism of liver slices, isolated liver mitochondria, and two types of ascites tumor cells, as well as on ion transport in liver slices. In liver slices, 0.2 to 1.0 mM SKF-525A caused an initial stimulation of O_2 uptake which was followed, at 0.5 to 1.0 mM, by a progressive inhibition of O_2 consumption, a fall of slice ATP content, and a reduced transport of K^+, Na^+ and Ca^{2+}. In isolated mitochondria, we studied the effects of SKF-525A on the rate of respiration and on the oxidation–reduction responses of $NAD(P)^+$ and cytochrome *b* in the presence of various substrates. The results suggest that SKF-525A had three distinct actions on liver mitochondria, viz. an uncoupling action at low concentrations (0.02 to 0.17 mM); at higher concentrations (0.2 to 0.5 mM) an inhibition of the oxidation of $NAD(P)^+$-linked substrates, exerted close to the substrate level; also at 0.2 to 0.5 mM, a less effective inhibition of electron transfer at a point between cytochrome *b* and O_2 in the electron-transfer chain. Experiments on O_2 consumption and cytochrome *b* oxidation–reduction changes in ascites cells showed only the first two of these effects in the intact tumor cells. We conclude that inhibition of mitochondrial energy-conserving reactions by SKF-525A can have a marked influence on energy-requiring aspects of liver-cell metabolism, one example of which is inhibition of cation active transport.

The agent SKF-525A (β-diethylaminoethyldiphenylpropyl acetate · HCl) is widely studied as an inhibitor of drug metabolism in the liver, having been used for this purpose in a variety of experimental preparations from intact animals to isolated microsomes [1]. The work on whole cells often assumes that the agent has a rather high degree of specificity towards the mixed function oxidases although a number of other effects of the drug are known. Thus, SKF-525A has been found to inhibit the membrane-transport of amino acids and their incorporation into proteins [2, 3], the synthesis of cholesterol [4], and the reabsorption of Na^+ and Cl^- by the kidney [5]; it leads to a fall of liver glycogen content [6] and affects the osmotic [7, 8] and excitable properties [9] of cell membranes.

An early finding was that SKF-525A affected mitochondrial respiration. Thus, Kensler *et al* [10] noted partial inhibition of cytochrome oxidase and succinoxidase preparations which, in contrast to the effects on microsomal oxidative enzymes, could be reversed by addition of a non-specific protein (serum albumin). Cooper, in unpublished work noted in Ref. 11, observed inhibition of the oxidation of carboxylic acids. Whitehouse [12] found that SKF-525A uncoupled oxidative phosphorylation in isolated liver mitochondria and suggested that this might contribute to the inhibition of cholesterol synthesis. A reduction of ATP synthesis could similarly contribute to a number of the other effects of the drug noted above, including inhibition of kidney function and alteration of membrane properties. However, Cooper *et al.* [11] confirmed the observation [10] that inhibition of mitochondrial respiration by SKF-525A was relieved non-specifically by proteins, and they concluded that the high intracellular protein content would probably prevent the inhibition of mitochondrial activity in intact cells.

In preliminary experiments, we noted that SKF-525A inhibited liver-slice respiration [13]. We now describe experiments which show that this drug has marked effects on mitochondrial energy-conserving metabolism at concentrations similar to those required for the inhibition of mixed function oxidases. The consequent effects on energy provision can have important consequences for cell activity, as indicated by an inhibition of cation transport. A preliminary report of some of these findings has been published [14].

METHODS

Experiments with liver were carried out on male, albino rats of Wistar strains (obtained in Philadelphia from Zivic–Miller, and in Rome from the colony of the Università Cattolica). Some of the rats were pretreated with phenobarbital in order to induce mixed function oxidases of the liver, the drug being administered in the drinking water for 5 days at a concentration of 1 mg/ml. The experimental procedures for the preparation and incubation of liver slices have been described previously [15]. The experiments included preincubation of the slices for 90 min at 1°, and appropriate concentrations of

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SKF-525A were added to the medium no more than 30 min after the start in order to allow equilibration with the tissue water. During the preincubation, the slices gained Na^+ and Ca^{2+} and lost K^+. Upon subsequent incubation at 38° in oxygenated medium, the changes of ionic content were largely reversed, the net increase of K^+ content and loss of Na^+ and Ca^{2+} providing measures of ion-transporting activity. The incubation medium contained (mM): Na^+, 156.0; K^+, 5.0; Ca^{2+}, 1.2; Mg^{2+}, 1.0; Cl^-, 159.0; SO_4^{2-}, 1.0; phosphate, 5.0; and Tris, 5.0 (pH 7.4). Respiration during incubation at 38° was measured by manometric methods, readings being taken at 10-min intervals, after an initial 10 min of equilibration. After incubation, the slices were collected and analyzed for ionic and ATP contents as described previously [15].

Rat liver mitochondria were isolated and studied for O_2 uptake as described by Johnson and Lardy [16], using a Clark-type electrode. The basic medium used for isolation and incubation of the mitochondria contained (mM): mannitol, 225; sucrose, 75; EDTA, 0.2; and Tris–HCl, 20 (pH 7.4); this is referred to as "MSE–Tris". Spectrophotometric measurements of nicotinamide nucleotides and cytochrome *b* in the mitochondria were performed at room temperature in the dual wavelength, split-beam Aminco–Chance spectrophotometer, at 340–375 nm and 430–410 nm respectively.

Experiments with ascites cells were carried out with Ehrlich hyperdiploid (H) and Ehrlich hyperdiploid Lettre (ELD) ascites cells. The cells were maintained by weekly i.p. transplantation in albino Swiss mice and harvested 6–8 days after inoculation. The peritoneal fluid was washed in an isotonic medium (154 mM NaCl, 6.2 mM KCl, 11 mM sodium phosphate, 10 mM Tris–HCl; pH 7.4) and, when slightly hemorrhagic, carefully freed from hemoglobin by differential centrifugation. The packed cells were then resuspended in the same medium to a final concentration of dry weight which depended on the experiment to be performed. For measurements of O_2 consumption, cell suspensions (3 ml) were incubated for 60 min at a concentration of 8–15 mg dry wt/ml in Warburg manometric flasks. Spectrophotometric measurements were carried out as for mitochondria in experimental cuvettes containing a total volume of 2.5 ml.

SKF-525A was a gift from Smith, Kline & French Inc., Philadelphia, PA, and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was supplied by Dr. R. B. Beechey of Shell Research Ltd.

The rather poor aqueous solubility of SKF-525A at neutral pH led us to compare different methods of preparing the experimental solutions. For all experiments with liver slices and some with mitochondria, the drug was dissolved in the appropriate incubation medium to give a stock solution of 10 mM, by addition of minimal amounts of HCl, from which the final concentrations were prepared. In the case of the experiments with slices, the pH of the final Ringer solutions was then carefully adjusted to 7.4. For work with mitochondria, the final solutions had somewhat differing pH values, as follows: 0.1 mM SKF-525A, final pH 7.33; 0.2 mM, pH 7.28; and 0.5 mM, pH 7.10. In this case the pH was not adjusted, and control experiments (without SKF-525A) were therefore run at pH 7.1 as well as at pH 7.4. The alternative method was to dissolve the drug in ethanol to give a stock solution of 50 mM, in which case ethanol was added to a concentration of 1% (v/v) in all experimental cuvettes. These two stock solutions are referred to as "aqueous" and "ethanolic" respectively.

RESULTS

Liver slices. The effect of SKF-525A on the time-course of O_2 consumption by liver slices from control and phenobarbital-induced rats is illustrated in Fig. 1. In the control tissues, the rate of respiration during the first 10-min period of observation increased with the concentration of the drug, the increase being statistically significant ($0.05 > P < 0.02$) at each concentration of 0.2 mM or greater. Subsequently, 0.5 and 1.0 mM SKF-525A caused a progressive decrease of O_2 consumption (Fig. 1). Liver slices from phenobarbital-treated rats were less sensitive to the drug; the early stimulation of O_2 consumption was not statistically significant while the later inhibition was only given by the highest concentration used (1.0 mM) and was less marked.

The inhibition of O_2 uptake in the slices could be due to inhibition of either microsomal or mitochondrial O_2 consuming reactions. To test the contribution of mitochondrial inhibition, we assayed the ATP content and net lactate production of the slices. In slices from control rats, these variables were little affected by SKF-525A concentrations up to 0.2 mM, but at higher concentrations ATP was reduced and lactate production was stimulated significantly (Table 1). This suggests that at least the two highest concentrations of SKF-525A inhibited mitochondrial oxidative metabolism, the increased lactate formation being due to a Pasteur effect. As was the case for O_2 consumption, the ATP content and, especially, the lactate production by slices from phenobarbital-induced livers were less sensitive to SKF-525A (Table 1).

The alterations of energy metabolism in slices caused by SKF-525A were accompanied by inhibition of the energy-dependent accumulation of K^+ and extrusion of Na^+ which occur when liver slices are incubated at 38° after preincubation at 1° [15, 17]. The effect of drug concentration on these transport processes was approximately parallel to its effect on the rate of respiration during the final 10 min of incubation (Fig. 2). The final rate is the relevant observation in this case, since the ionic contents of the slices were determined upon termination of the incubation. Similar results were obtained with slices from control rats and phenobarbital-treated rats (Fig. 2, a and b). Liver slices at 38° also extrude Ca^{2+} by means of an energy-dependent transport process [18, 19] and this, too, was inhibited by SKF-525A (Table 2).

Liver mitochondria. To elucidate the mechanism of action of SKF-525A on cell respiration, we studied its effects on mitochondria isolated from the livers of control rats. In the absence of SKF-525A, the mitochondria showed respiratory control ratios of 4–6 upon addition of ADP. Increasing concentra-

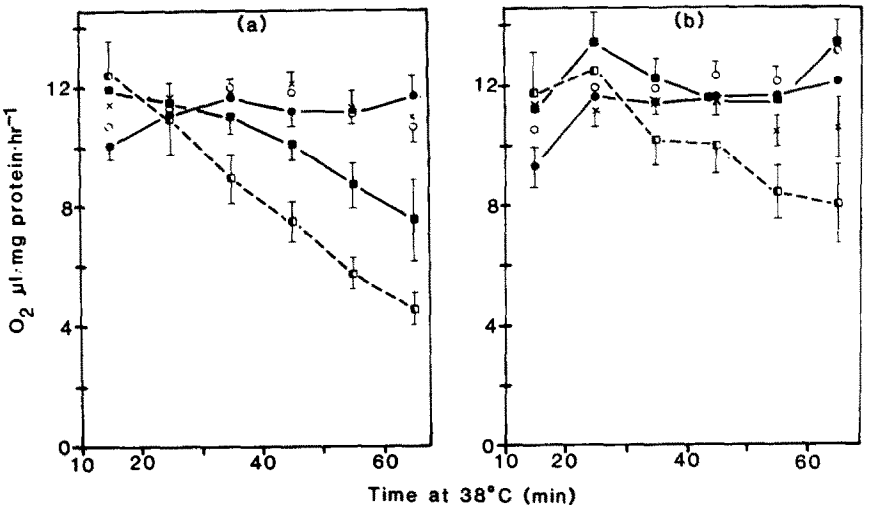


Fig. 1. Effects of SKF-525A on the rate of respiration during the course of incubation of liver slices prepared from (a) control and (b) phenobarbital-treated rats. Phenobarbital treatment is described in Methods. The slices were incubated at 38° after preincubation at 1° for 90 min in phosphate-buffered Ringer solution containing various concentrations of SKF-525A. The incubation was carried out in a Warburg manometric apparatus, after gassing with O_2. Readings of O_2 uptake were taken every 10 min after 10-min equilibration. Concentrations of SKF-525A (mM) were: (—●—) none; (○) 0.1; (×) 0.2; (—■—) 0.5; and (--■--) 1.0. The points at 0.1 and 0.2 mM have not been connected by lines, for the sake of clarity. Each point is the mean ± S.E.M. of eleven to seventeen observations.

tions of the drug caused a progressive decrease of the respiratory control ratio; stimulation by ADP was completely abolished by 0.5 mM SKF-525A, when glutamate *plus* malate was substrate (Fig. 3), and by 0.17 mM with pyruvate *plus* malate (Fig. 4). At the lowest concentrations of SKF-525A, the reduction of the respiratory control ratio was solely due to an increase of the rate of respiration in state 4, suggesting that the drug uncoupled oxidative phosphorylation. At higher concentrations the rate of state 3 respiration was reduced and above 0.2 mM SKF-525A the rate in state 4 also fell, results which indicate that the transfer of electrons into or through the respiratory chain was inhibited.

The action of SKF-525A as an inhibitor of electron transfer was most effective against NAD^+-linked substrates. For example, in the presence of excess ADP (2 mM), the O_2 consumption with glutamate *plus* L-malate (4 mM each) was inhibited 67% by 0.5 mM SKF-525A (reduced from 150 to 47 nmoles/min); but it was inhibited by only 27% (reduced from 210 to 131 nmoles/min) with 4 mM succinate as substrate and by 31% (reduced from 37 to 25 nmoles/min) with 40 µM tetramethylene-*p*-phenylenediamine (TMPD). In the last instance, antimycin A (5 µg/ml) was present to inhibit oxidation of endogenous substrates.

To examine further the effects of SKF-525A on the respiratory chain, we studied the oxido-reduction levels of $NAD(P)^+$ and cytochrome *b* in different

Table 1. ATP contents and lactate production in liver slices incubated with various concentrations of SKF-525A*

Incubation	SKF-525A (mM)	Control rats			Phenobarbital-induced rats		
		ATP	Lactate	N	ATP	Lactate	N
1°	0 & 1.0	5.97 ± 0.74		11	5.12 ± 0.41		12
38°	0.0	6.31 ± 0.56	68.4 ± 9.0	17	5.56 ± 0.40	53.1 ± 4.5	15
	0.1	5.52 ± 0.41	73.0 ± 9.0	17	5.78 ± 0.48	61.5 ± 6.6	15
	0.2	5.60 ± 0.72	77.8 ± 7.9	13	5.54 ± 0.54	48.2 ± 12.5	15
	0.5	2.98 ± 0.38†	95.2 ± 5.6‡	18	3.97 ± 0.40†	64.1 ± 7.8	18
	1.0	0.98 ± 0.15†	102.9 ± 12.5‡	7	1.20 ± 0.15†	78.1 ± 10.6‡	9

* Slices were preincubated for 90 min at 1° followed by 70 min of incubation at 38° in an oxygenated medium. They were collected at the end of this total incubation and assayed for ATP. At the same time, a sample of the medium was removed and assayed for lactate. The lactate was corrected for the quantity present at the end of preincubation at 1°, in order to assess the amount produced during incubation at 38°. The ATP contents of slices incubated at 1° with and without SKF-525A (1 mM) were not significantly different from each other and have been combined in the table. Values are means ± S.E.M. ATP contents are expressed as mmoles/kg protein and lactate as mmoles produced · kg protein^{-1} · hr^{-1}.

† $P < 0.01$ (Student's *t*-test).

‡ $P < 0.05$ (Student's *t*-test).

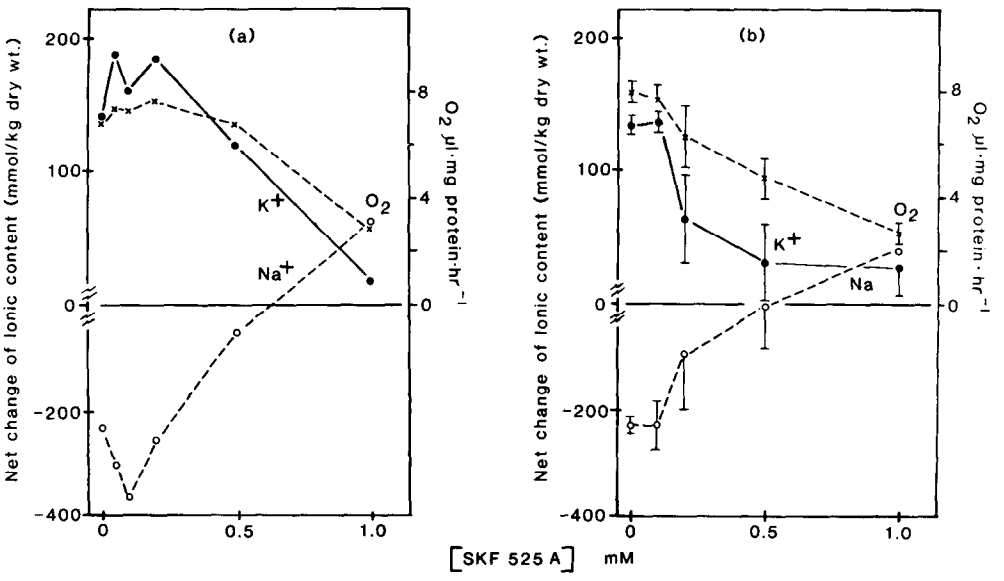


Fig. 2. Effect of increasing concentrations of SKF-525A on the net movements of Na^+ and K^+ in liver slices from (a) control and (b) phenobarbital-treated rats. Incubation conditions were as described in the legend of Fig. 1. Samples of slices were taken for analysis after the preincubation at 1° and again after completion of the further incubation for 70 min at 38°. The difference of ionic content between the two sets of samples represents the net uptake of K^+ or loss of Na^+ due to active transport [17]. The rate of respiration shown is that determined during the final 10 min of incubation, as this immediately preceded determination of the final ionic content of the slices. Each point is the mean of two observations in (a) and of four to six observations in (b). Key: (—●—) K^+; (--○--) Na^+; and (-- × --) QO_2.

metabolic states. The effects of various concentrations of SKF-525A were first studied, using the aqueous stock solution and performing control experiments at pH 7.1 as well as at 7.4 (see Methods).

At the start of each experiment (Fig. 5), the nicotinamide nucleotides of liver mitochondria utilizing endogenous substrate only (i.e. the left-hand portion of each trace) showed a rather similar tendency to become oxidized, irrespective of the concentration of SKF-525A. Addition of phosphate to control

Table 2. Effects of SKF-525A on the net extrusion of Ca^{2+} by liver slices*

SKF-525A (mM)	Extrusion of Ca^{2+} (mmoles/kg dry wt)	
	Control rats	Phenobarbital-treated rats
0.0	4.9	6.4 ± 0.8
0.05	8.4	
0.1	4.0	6.7 ± 1.1
0.2	6.5	5.3 ± 4.8
0.5	5.5	9.0 ± 1.4
1.0	1.5	−0.1 ± 1.5

* Net extrusion was determined as the difference between the Ca^{2+} content of liver slices analyzed after preincubation at 1° and of slices analyzed after further incubation for 60 min at 38°. SKF-525A was added to the medium, at the indicated concentrations, during the first 30 min at 1°. Treatment of rats with phenobarbital is described in Methods. Values are means ± S.E.M. For slices from control rats, N = 2; for phenobarbital-treated rats, N = 4–10.

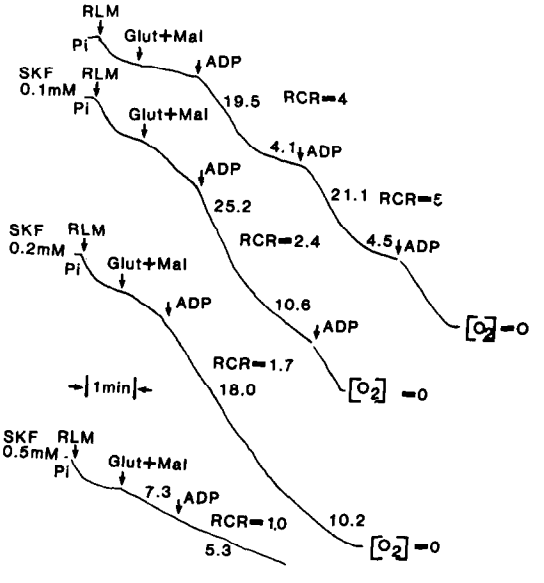


Fig. 3. Recording of the O_2 consumption of rat liver mitochondria in the presence of different concentrations of SKF-525A. Additions were as indicated by arrows: P_i, 4 mM potassium phosphate; RLM, rat liver mitochondria (2.9 mg protein/ml); G + M, 4 mM glutamate *plus* 4 mM L-malate; ADP, 400 µM adenosine diphosphate; SKF, SKF-525A added to the medium at the indicated concentrations, from the ethanolic stock solution, before all other additions. The numbers along the traces represent the rates of respiration at the adjacent point, nmoles O_2 · mg protein^{-1} · min^{-1}; RCR, respiratory control ratio [20].

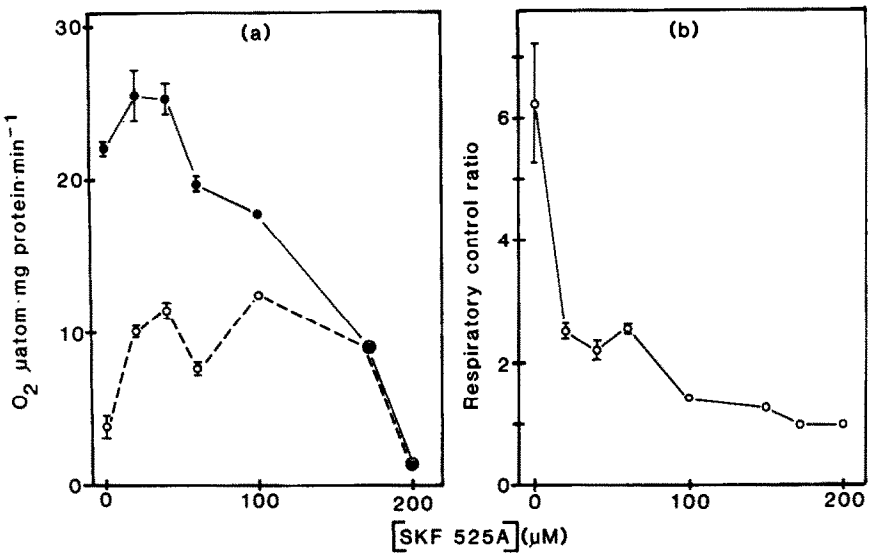


Fig. 4. Effects of SKF-525A concentrations on (a) O_2 consumption in states 3 and 4 and (b) respiratory control ratio, of rat liver mitochondria using pyruvate *plus* malate as substrates. The data were from experiments of the type seen in Fig. 3, except for the use of 1 mM pyruvate *plus* 1 mM L-malate instead of glutamate *plus* malate. Each point is the mean of two to six observations. In panel a, (●) state 3, and (○) state 4.

mitochondria always resulted in a reduction of $NAD(P)^+$, although in the control at pH 7.1 this was preceded by a rapid oxidation. At 40 µM, SKF-525A prevented the reductive effect of phosphate, while higher concentrations of the drug permitted continued oxidation.

Addition of glutamate *plus* malate, from which electrons enter the respiratory chain at the level of NAD^+, gave a rapid reduction which, however, was not maintained (Fig. 5a). When 40 or 200 µM SKF-525A was present (Fig. 5b), the substrates still induced a rapid movement of the trace towards

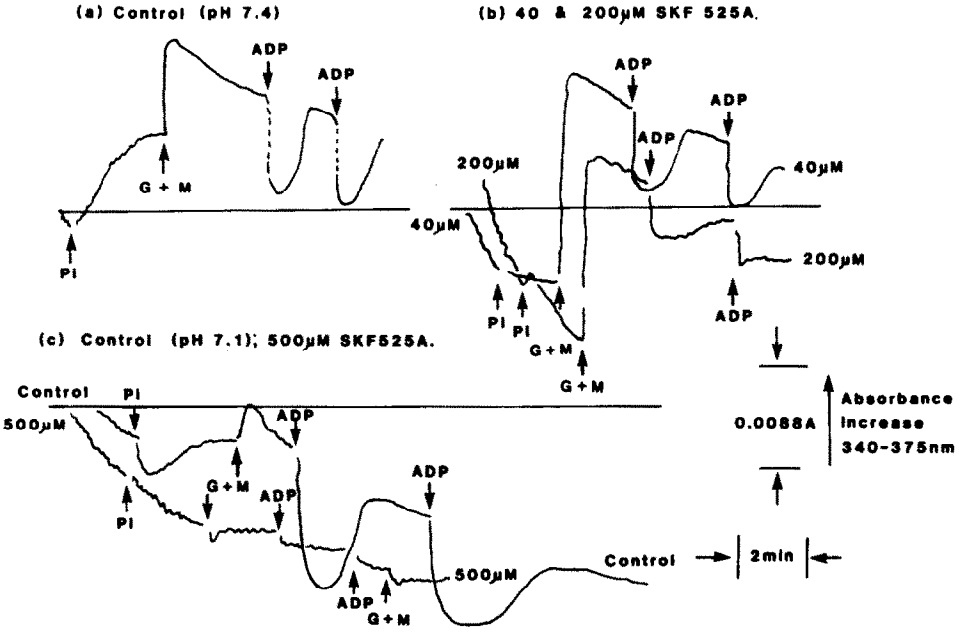


Fig. 5. Effects of SKF-525A on the oxido-reduction levels of nicotinamide nucleotides of rat liver mitochondria utilizing glutamate *plus* malate, in different metabolic states. Mitochondria (4.0 mg protein) were incubated in 2.5 ml MSE–Tris containing the indicated concentrations of SKF-525A; the latter was added from aqueous stock solution. Additions of metabolites and other inhibitors were made as follows: P_i, 4 mM potassium phosphate; G + M, 4 mM glutamate *plus* 4 mM malate; and ADP, 400 µM adenosine diphosphate. Control traces made at medium pH of 7.4 and 7.1 are illustrated. The dual-beam spectrophotometric tracings represent the difference of absorbance between 340 and 375 nm.

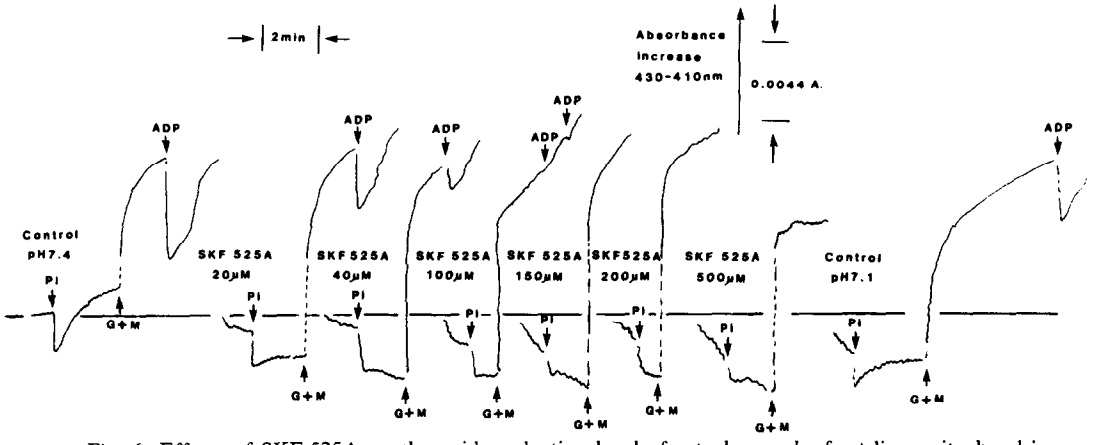


Fig. 6. Effects of SKF-525A on the oxido-reduction level of cytochrome *b* of rat liver mitochondria utilizing glutamate *plus* malate, in different metabolic states. Details of additions were as described in the legend of Fig. 5, except that the quantity of mitochondrial protein was 4.3 mg. The tracings are of the difference of absorbance between 430 and 410 nm.

reduction of $NAD(P)^+$. Indeed, the deflection was greater than in the control mitochondria, probably because the oxido-reduction level induced by phosphate was more oxidized in the presence of the drug. In the control medium at pH 7.1, the reduction of $NAD(P)^+$ upon addition of glutamate *plus* malate was less than at pH 7.4 (Fig. 5c), but it was totally abolished by 0.5 mM SKF-525A which, indeed, induced some oxidation (see also Fig. 7). Clearly, this concentration of the drug inhibited the transfer

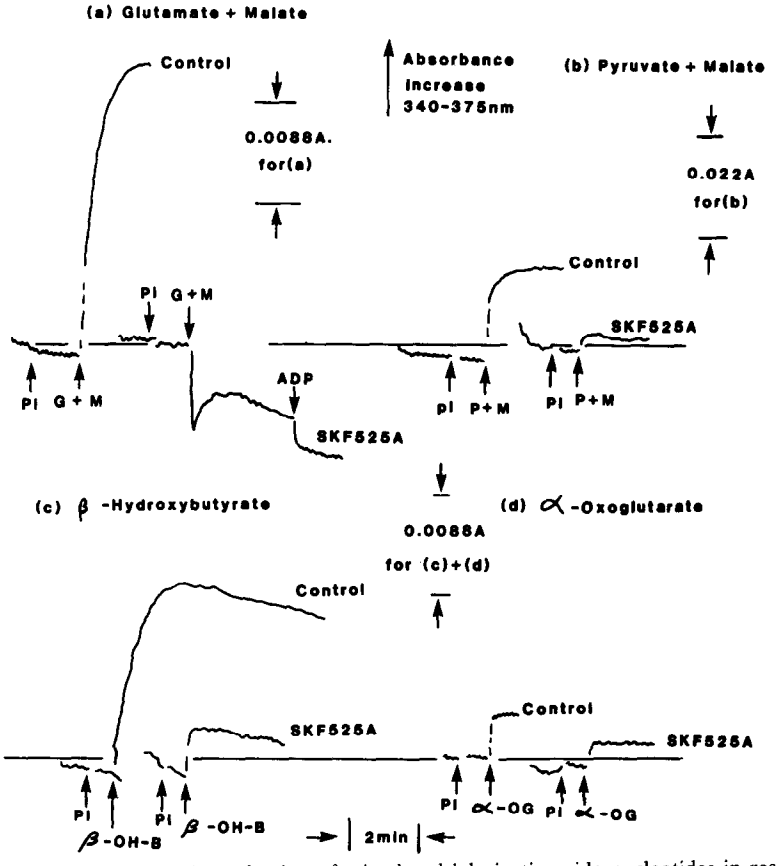


Fig. 7. Effects of SKF-525A on the reduction of mitochondrial nicotinamide nucleotides in response to different $NAD(P)^+$-linked substrates. In each tracing, 4.1 mg mitochondrial protein was added to 2.5 ml medium, with or without 0.5 mM SKF-525A. The latter was added from the ethanolic stock solution. Each pair of tracings illustrates the effect of substrate addition to mitochondria in the absence (control) or presence of SKF-525A. Substrates used were: (a) 4 mM glutamate *plus* 4 mM malate; (b) 4 mM pyruvate *plus* 4 mM malate; (c) 4 mM β-hydroxybutyrate; (d) 4 mM 2-oxoglutarate. The traces in b and d have been corrected for the non-enzymic change in absorbance caused by the substrate itself. P_i, 4 mM potassium phosphate. Absorbance difference, 340–375 nm.

of electrons from the substrates to $NAD(P)^+$.

The effects of phosphate on cytochrome *b* in the presence of increasing concentrations of SKF-525A were qualitatively similar to those on $NAD(P)^+$, a biphasic oxidation and reduction in the controls changing to a single phase of oxidation (Fig. 6). Addition of glutamate *plus* malate led to reduction of cytochrome *b* which, measured from the level attained with phosphate, was substantially greater in extent in the presence of 20–200 μM SKF-525A than in the absence of the drug. At 500 μM, SKF-525A substantially inhibited the reduction.

Addition of ADP to control mitochondria caused cycles of oxidation followed by reduction of both nicotinamide nucleotides and cytochrome *b*, but the presence of increasing concentrations of SKF-525A progressively blocked these changes (Figs. 5 and 6). The cycles of NAD(P)H were abolished completely at 500 μM SKF-525A and of cytochrome *b* at 100 μM, indicating a loss of respiratory control.

We next compared the inhibitory specificity of 0.5 mM SKF-525A towards a number of NAD^+-linked substrates; for convenience, we used drugs from the ethanolic stock solution. The substrates glutamate *plus* L-malate, pyruvate *plus* malate, β-hydroxybutyrate, and 2-oxoglutarate (Fig. 7) gave varying degrees of reduction of $NAD(P)^+$ in control runs but in each case the deflection of the trace was reduced drastically (by 50–80%) in the presence of SKF-525A.

To elucidate the site at which SKF-525A inhibits succinate-supported respiration, we compared its effects on the reduction of cytochrome *b* by electrons from succinate with the actions of rotenone, which inhibits electron transfer between NADH and cytochrome *b*, and of antimycin A, which inhibits

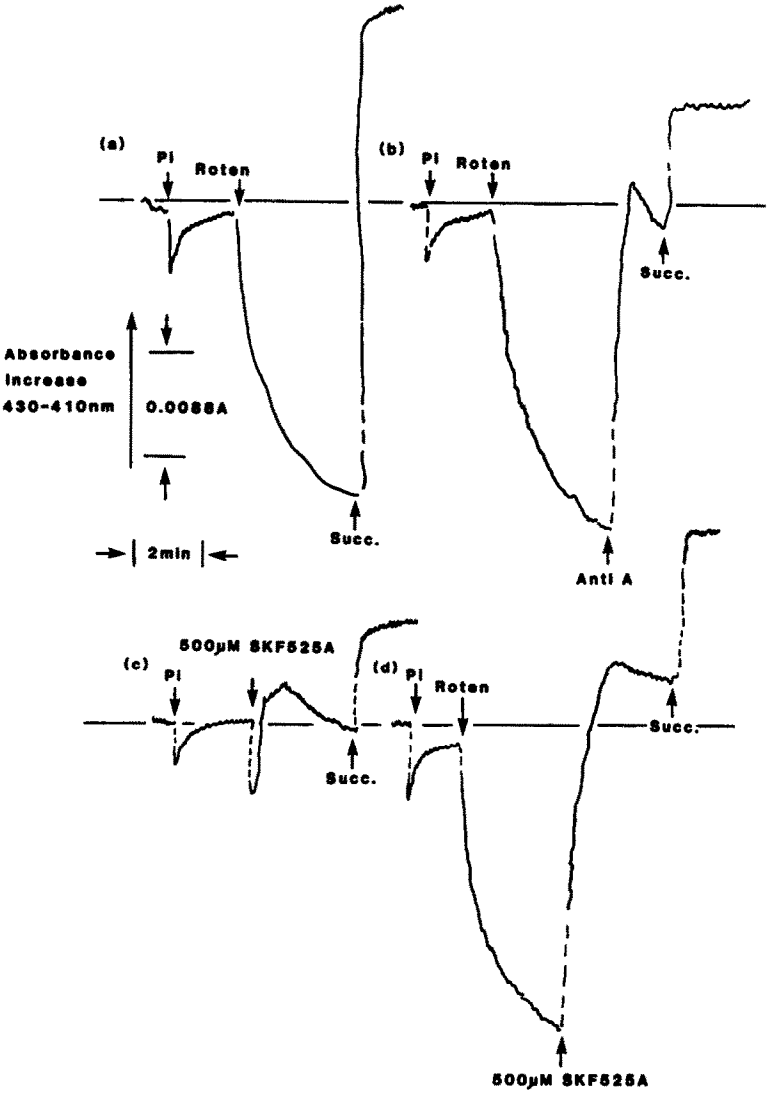


Fig. 8. Effects of inhibitors on the reduction of cytochrome *b* in rat liver mitochondria. Mitochondrial protein in each cuvette was 4.3 mg. SKF-525A was added to the indicated, final concentrations from ethanolic stock solution. Final concentrations of additions were: P_i, 4 mM potassium phosphate; Succ, 4 mM succinate; Roten, 4 μM rotenone; and Anti A, 3.6 μg/ml antimycin A. Traces show the absorbance difference between 430 and 410 nm.

between cytochromes *b* and *c*. Rotenone caused a large oxidation of cytochrome *b* by inhibiting electron transfer from endogenous substrates, and the oxidation was reversed upon addition of succinate (Fig. 8a). The effect of rotenone was also reversed by antimycin A (Fig. 8b), implying that some electrons reached cytochrome *b* from endogenous, flavoprotein-linked substrates; addition of exogenous succinate further increased the level of reduction. The action of SKF-525A (0.5 mM) in the presence of rotenone was entirely analogous to that of antimycin (Fig. 8d). The extent of cytochrome reduction induced upon addition of succinate was nearly identical under three conditions, viz. in the presence of rotenone *plus* antimycin A, in the presence of SKF-525A alone, and in the presence of rotenone *plus* SKF-525A (Fig. 8, b, c and d respectively). We conclude that SKF-525A had no effect on the transfer of electrons from succinate to cytochrome *b* but, like antimycin A, prevented electron transfer from cytochrome *b* to O_2. Such a site of action is further indicated by the observation (not shown) that 0.5 mM SKF-525A added before rotenone prevented the oxidation of cytochrome *b* which the latter agent induced.

Ascites cells. The respiration of hyperdiploid (H) cells utilizing only endogenous substrates was stimulated slightly by 0.1 and 0.2 mM SKF-525A while an inhibition was seen with concentrations of 0.3 mM and above (Fig. 9a), results which agree well with those obtained from liver slices. These cells showed a Crabtree effect upon addition of glucose (Fig. 9b) and this was released by SKF-525A at concentrations up to 0.3 mM, an effect to be anticipated from an uncoupling action of the drug. Analogous results were obtained with mutant Lettre (ELD) cells (Fig. 9, c and d), but with small quantitative differences in that these cells showed a greater sensitivity to the inhibitory action of the drug.

In Fig. 10a, the respiration of ELD cells was stimulated by succinate. The endogenous respiration was almost completely inhibited both by SKF-525A at 0.5 mM (Fig. 10b) and by rotenone (Fig. 10c), but in each case the addition of succinate gave an extra O_2 consumption which was similar to that in the absence of inhibitors. Figure 10e shows that arsenite, which inhibits 2-oxoglutarate dehydrogenase, had a completely different effect from SKF-525A in that it markedly inhibited the stimulation of respiration by succinate. It thus seems unlikely that SKF-525A acted as an inhibitor of the citric acid cycle. These findings agree with those from liver mitochondria in suggesting that 0.5 mM SKF-525A reduces the transfer of electrons from NAD-linked substrates, but differ from them in that SKF-525A did not appear to affect the transfer of electrons from succinate.

In confirmation of these conclusions, we found that 0.1 and 0.5 mM SKF-525A, like rotenone (8 μM), caused a large oxidation of cytochrome *b* in ELD ascites cells. Addition of glucose, briefly stimulating consumption of ATP and thus inducing state 3 in the mitochondria, caused a small, temporary oxidation of cytochrome *b* while subsequent addition of succinate caused its marked reduction (not illus-

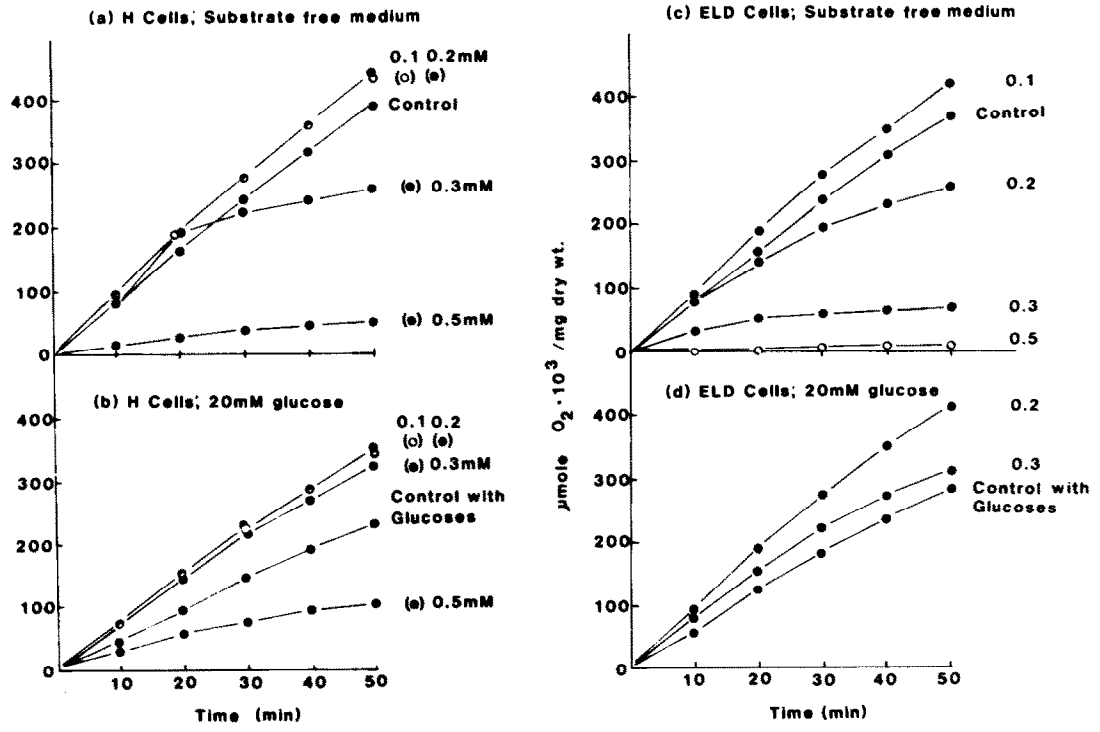


Fig. 9. Effects of SKF-525A on respiration of ascites cells in the presence and absence of glucose. Oxygen consumption was determined manometrically, readings being taken every 10 min after an initial 10-min equilibration. The concentrations of SKF-525A are indicated to the right of each line. Each point is the mean of one to three observations. The drug was added from ethanolic stock solution.

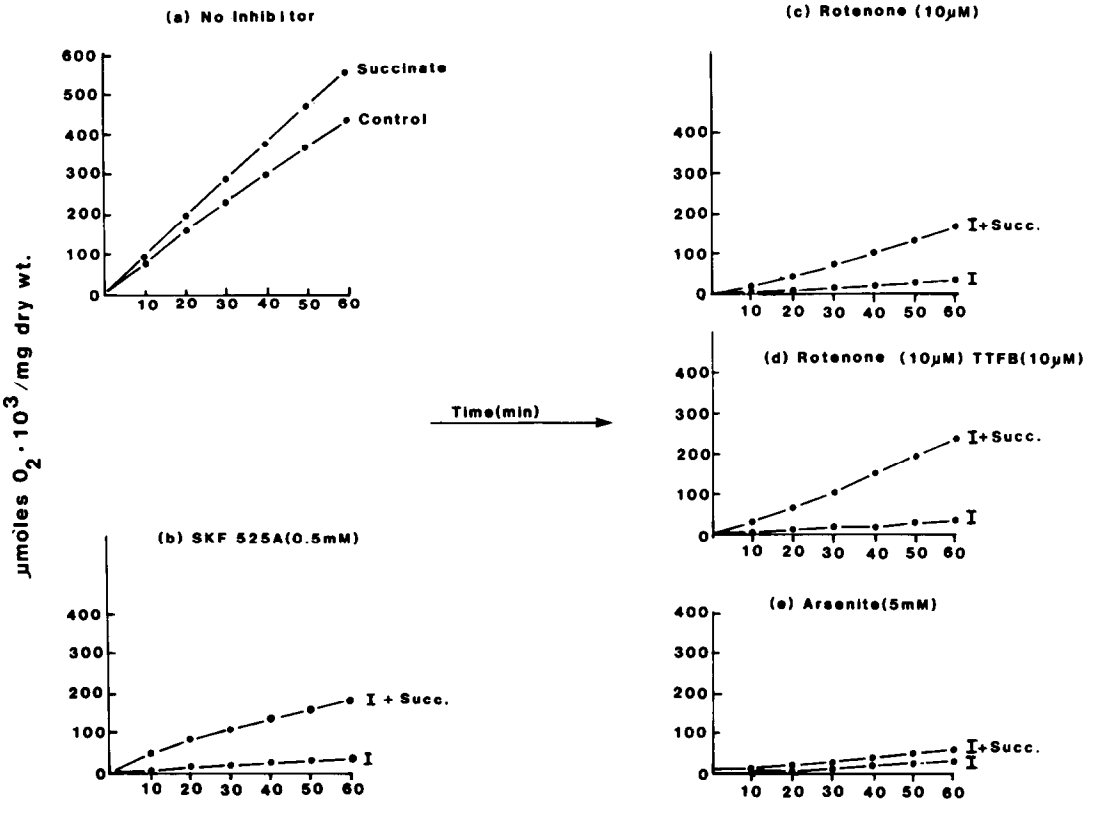


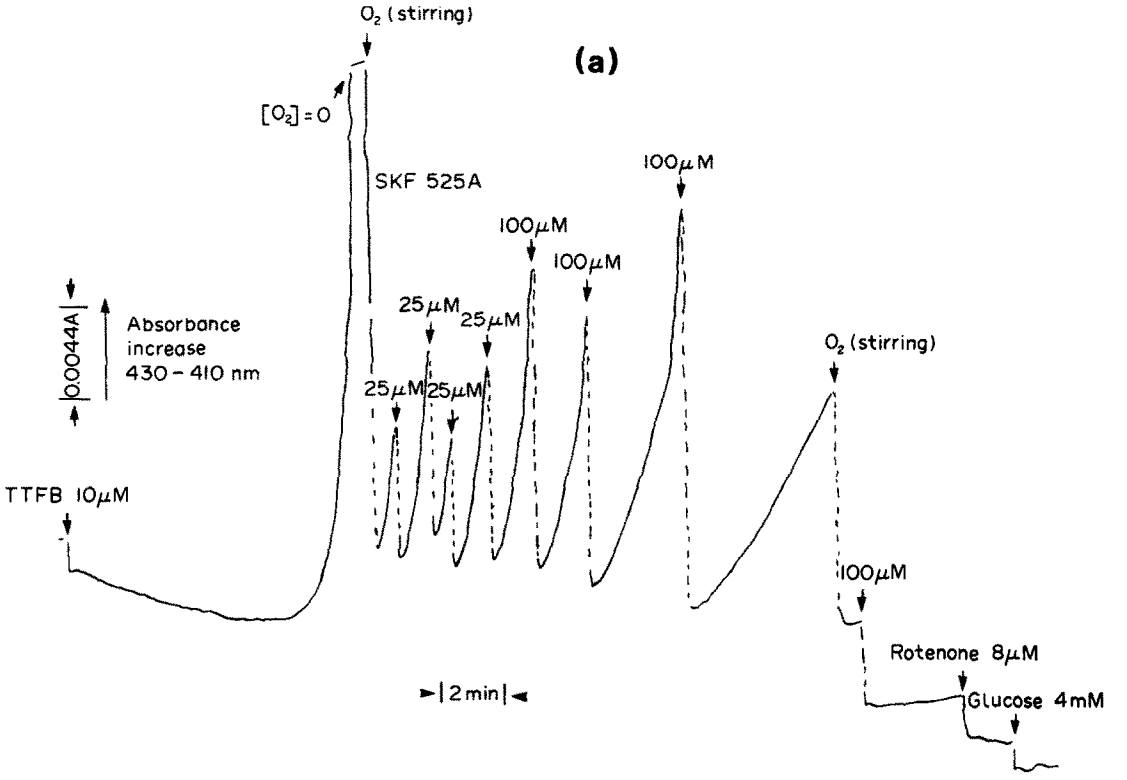
Fig. 10. Effects of SKF-525A and other inhibitors on the O_2 consumption by ELD ascites cells utilizing succinate as substrate. Panel (a) illustrates the effect of 10 mM succinate on O_2 consumption compared to a control with endogenous substrate only. All other panels compare the O_2 consumption in the presence of the indicated inhibitor alone (I) with that in the presence of the inhibitor *plus* 10 mM succinate (I + Succ). Oxygen uptake was determined manometrically, as in Fig. 9.

trated). Another approach to the same point is illustrated in Fig. 11, where the medium was made anaerobic by addition of TTFB, and cytochrome *b* was consequently reduced. Upon introduction of a limited amount of O_2 into the medium, by a brief stirring, the cytochrome was rapidly oxidized but then became reduced again by electrons derived from endogenous substrates. The reduction curve was biphasic (Fig. 11a). The initial, slower phase we have attributed to electron-flow from substrate to cytochrome *b* while the second, more rapid phase was determined by the onset of anaerobiosis. Subsequently, small increments of SKF-525A were stirred into the medium together with the O_2, and the rate of the first phase of reduction of cytochrome *b* became progressively slower as the concentration of the drug increased (Fig. 11b); this is compatible with an increasing inhibition of the transfer of electrons from NAD^+-linked endogenous substrates to cytochrome *b*. The inhibition was complete when the sum of additions of SKF-525A gave a total medium concentration of 0.5 mM, and cytochrome *b* then remained in a highly oxidized state which was only slightly affected by subsequent additions of rotenone and glucose.

DISCUSSION

Our results with liver slices and liver mitochondria agree in showing at least two effects of SKF-525A on respiration, namely an uncoupling action at low concentrations and an inhibition of O_2 consumption at higher concentrations. The uncoupling effect is manifested as an early stimulation of respiration in the slices (Fig. 1), and in the mitochondria as stimulation of state 4 respiration and loss of respiratory control (Figs. 3 and 4) as well as by abolition of the oxidation–reduction response of electron carriers to ADP (Figs. 5 and 6). The concentrations of SKF-525A required for the initial uncoupling effect in the slices tended to be rather greater than those required in the isolated mitochondria, but such a comparison is rather arbitrary in view of the time-dependence of the effects of the drug on the slices.

More detailed studies of the mechanism of action of SKF-525A were restricted to an examination of the point, or points, at which high concentrations (0.5 mM) acted to inhibit mitochondrial respiratory activity. The preliminary observation that O_2 consumption with NAD^+-linked substrates was inhibited markedly more than that with either succinate or TMPD indicated a point of action prior to the entry of electrons from succinate into the respiratory chain. Studies of the electron carriers confirmed this conclusion and showed that the site of this action is at, or before, that at which $NAD(P)^+$ becomes reduced. The evidence is 3-fold, viz. (i) 0.5 mM SKF-525A diminished the reduction of cytochrome *b* which was



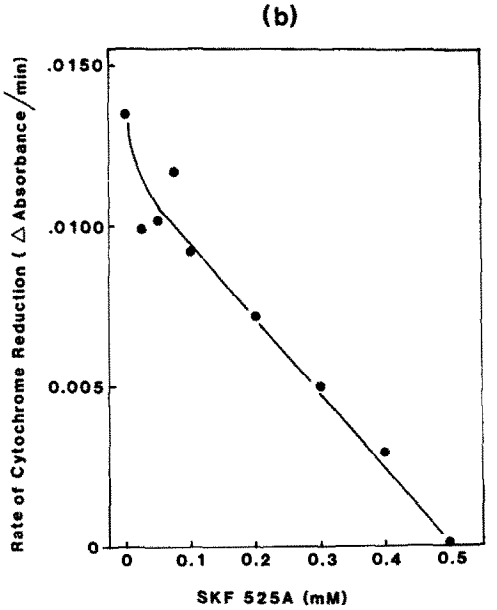


Fig. 11. Oxidation–reduction changes of cytochrome *b* in ELD ascites cells, illustrating the effect of increasing concentrations of SKF-525A on the rate at which the cytochrome becomes reduced by endogenous substrates. (a) Dual-beam spectrophotometric trace (430–410 nm). Anaerobiosis was first established in the cuvette (containing 4.5 mg dry wt cells/ml) in the presence of 10 μM TTFB. Limited amounts of O_2 were then introduced into the medium from the atmosphere by stirring; this resulted in a rapid oxidation of cytochrome *b* (downward deflection) followed by its reduction by electrons from endogenous substrates. Amounts of SKF-525A were introduced together with stirring to oxygenate the medium at each arrow; the increase in drug concentration in the medium upon each such addition is indicated. (b) Plot of the rate of cytochrome *b* reduction versus accumulated concentration of SKF-525A in the medium, determined from the initial slopes of reduction after each addition of O_2 in (a).

caused by the NAD^+-linked substrates, glutamate + malate (Fig. 6); (ii) it prevented, or greatly diminished, the reduction of $NAD(P)^+$ by these, and several other, NAD^+-linked substrates (Figs. 5 and 7); and (iii) it prevented the reduction of $NAD(P)^+$ and cytochrome *b* upon addition of inorganic phosphate to mitochondria utilizing endogenous substrates (Figs. 5 and 6). The reason for the reduction of $NAD(P)^+$ upon addition of phosphate in the absence of SKF-525A is not entirely clear, but we suggest that phosphate stimulated the substrate-level oxidation in the citric-acid cycle. The blocking by SKF-525A of the reduction of $NAD(P)^+$ could be due to direct inhibition of the $NAD(P)^+$-dependent dehydrogenases, or to an effect on the permease systems facilitating passage of the substrates through the inner mitochondrial membrane. However, the latter explanation cannot account for the finding (No. iii, above) that SKF-525A prevented the reduction of $NAD(P)^+$ in the presence of endogenous substrates only. The failure of the drug to inhibit transfer of electrons from succinate to cytochrome *b* also argues against an effect on permeases since this substrate also enters mitochondria by a malate-dependent transport mechanism.

Our measurements of cytochrome *b* in the mitochondria show that there must also be a second point at which high concentrations of SKF-525A inhibit the transfer of electrons. In particular, the observation that 0.5 mM SKF-525A induced a reduction of cytochrome *b* when added after rotenone, but prevented oxidation of cytochrome *b* when added before rotenone, indicates that SKF-525A inhibits electron transfer between cytochrome *b* and O_2. Since mitochondrial O_2 consumption with either succinate or TMPD as substrate was inhibited to a similar extent (approx. 30%) by SKF-525A, it seems likely that the site of this action of the drug lies between cytochrome *c* and O_2.

The results with ascites cells support the conclusion that SKF-525A uncouples oxidative phosphorylation at low, and inhibits electron transfer from $NAD(P)^+$-linked substrates at higher, concentrations. But SKF-525A had no effect on electron transfer between cytochrome *b* and O_2. The reason for this difference from isolated liver mitochondria could reside in a difference between the mitochondria of ascites and liver cells or in a difference between isolated mitochondria and those *in situ*.

While SKF-525A at a concentration of 0.5 mM thus clearly inhibits oxidative reactions in liver mitochondria, it cannot be readily concluded that these effects, rather than the uncoupling action of lower concentrations, account for the effects of 0.5 and 1.0 mM SKF-525A on energy metabolism in the slices. Especially, the observation that these concentrations of SKF-525A showed a time-lag before producing their inhibition of O_2 consumption in the slices casts doubt on the role of direct inhibition of mitochondrial electron transfer reactions. The lag is unlikely to be due to a difficulty of penetration of the drug to its site of action in the slices, for two reasons. First, the drug was in contact with the slices for at least 60 min preincubation at 1° and, second, significant stimulatory effects on respiration were observed during the first observation period of O_2 consumption, with concentrations as low as 0.2 mM, showing that the drug had already reached the mitochondria. It is more likely that the uncoupling action of SKF-525A led to a reduction of cellular ATP levels and that this progressively reduced the possibility for activation of endogenous substrates (e.g. fatty acids). In any case, it is clear that the net effect was a marked reduction of ATP in the slices which could have important consequences for cellular function. In our experiments, these effects included the inhibition of two distinct ion transport systems, namely that responsible for the ouabain-sensitive, coupled transport of Na^+ and K^+ [17], and that bringing about the energy-dependent extrusion of Ca^{2+} [18, 19]. The concentrations of SKF-525A at which these effects were observed in liver slices fall within the range at which the drug has been used to inhibit mixed function oxidase activity in preparations of intact liver cells such as perfused liver [1], liver slices [11, 21], and isolated hepatocytes [1, 8]. It would appear necessary, in interpreting such experiments, to take into consideration the possibility that the treated cells are markedly abnormal with respect to energy metabolism and metal ion homoeostasis.

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