72-5078

PILCHMAN, Peter, 1944-
THE ACTION OF SKF525-A AND RELATED COMPOUNDS ON ISOLATED RAT LIVER MITOCHONDRIA.

The City University of New York, Ph.D., 1971
Biology

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1971

THE ACTION OF SKF525-A AND RELATED COMPOUNDS ON ISOLATED RAT LIVER MITOCHONDRIA

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1971

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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The author expresses his thanks to the many people who took an interest in this work: Professor Edward Balboni, who helped in the experimental designs; Miss Ursula Behrens, who took the electron micrographs; Dr. Harvey Penefsky, who helped greatly in the interpretation of the data; Mr. Victor Jules, who expedited the ordering of supplies; Jim Paterniti, who drew the figures ; and Mrs. Becky Field, who cheerfully typed the manuscript. The author is indebted to Dr. Sheldon Aaronson for his continuing practical and realistic advice, his encouragement, and, most importantly, his great patience.

Finally, without the sacrifices of my aunt, Fran Arcara, this work could never have been done.

To Harry Schachner,

For His Love of Children

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ABBREVIATIONS

1.	ADP	Adenosine - 5' - diphosphate
2.	ATP	Adenosine - 5' - triphosphate
3.	m-C1-CCP	Carbonyl cyanide m-chlorophenylhydrazone
4.	Co Q	Ubiquinone
5.	DEAE	Diethylaminoethanol
6.	DMAE	Dimethylaminoethanol
7.	DNP	2,4 - dinitrophenol
8.	DPEAA	Diphenylethylacetic acid
9.	FCCP	Carbonylcyanide p - trifluoromethoxyphenylhydrazone
10.	FP_1	NADH dehydrogenase
11.	FP_2	Succinate dehydrogenase
12.	HEPES	N-2-Hydroxyethylpiperazine N'-2-ethanesulfonic acid
13.	NAD	Nicotinamide adenine dinucleotide
14.	PCA	Perchloric acid
15.	PMS	Phenazine methosulfate
16.	Pr	Protein
17.	TCA	Trichloroacetic acid
18.	TMPD	Tetramethyl - p - phenylenediamine
19.	TTFB	Tetrachlorotrifluoromethyl benzimidazole

INTRODUCTION

The initial interest in searching for pharmacologic agents that would lower human, serum cholesterol levels (hypocholesteremic agents) was based on the implication of elevated serum lipid levels as one cause of the disease atherosclerosis.[1] A large number of hypocholesteremic agents have been synthesized since the discovery of the first such agent, alphaphenyl butyrate.[2] The hypocholesteremic agent triparanol was discovered a few years later.[3] The block in the cholesterol biosynthetic pathway, brought about by triparanol, was localized at the reactions saturating side chain double bonds on the sterol molecule.[4] The clinical use of triparanol was discontinued in 1962 due to its harmful side effects.[5] When it was understood that as the serum cholesterol concentration fell, the serum desmosterol concentration rose, and that desmosterol was deposited in atherosclerotic lesions as rapidly as cholesterol, the rationale for the use of triparanol in preventing atherosclerosis became questionable.[1]

In an effort to predict possible harmful side effects in humans and to determine the cellular sites of action of certain hypocholesteremic agents, Aaronson and colleagues studied the effects of the drugs on the respiration and multiplication of a number of microbial systems.[6,7,8,9,10] The respiration and multiplication of protozoa were inhibited by a large number of hypocholesteremic agents and oleic acid annulled the inhibition of certain of these compounds.[6,7,8,9,10] In mammalian

systems hypocholesteremic drugs had been shown to interfere with steroid biosynthesis, therefore it was expected that sterols and sterol precursors would annul the inhibition since some of the protozoa tested synthesized sterols.[4] As a result of oleic acid annullment of drug action and lack of annullment by sterols and their precursors[6], it was proposed that these drugs inhibited the biosynthesis or function of unsaturated fatty acids in protozoa.[6] Procaryote systems, which do not synthesize much, if any, sterols, showed similar effects in that multiplication could be inhibited by several hypocholesteremic agents and that this inhibition could be annulled by oleic acid and interestingly, the steroid ergosterol.[8]

Among the hypocholesteremic agents studied in Aaronson's laboratory were B-diethylaminoethyl-diphenylpropylacetate·HC1 (SKF525-A), two molecules representing the two parts of SKF525-A (diphenylpropylacetic acid, SKF2314, and diethylaminoethanol, DEAE), and molecules that could be looked on as analogs of SKF525-A (Table 1). Using the protozoan Ochromonas danica, Aaronson found that the diphenylpropylacetic acid part of the SKF525-A molecule (SKF2314) was the active part of the molecule inhibiting multiplication; the diethylaminoethyl part of the SKF 525-A molecule (DEAE) was without effect.[11] It was suggested, on the basis of the difference of annulling agents and certain other criteria, that SKF525-A and SKF2314 acted on different metabolic

sites.[11]

Whitehouse reported in 1964 that several hypocholesteremic agents, SKF525-A among them, uncoupled rat liver mitochondria.[12] This claim was based on the inhibition of the P/O ratio during succinate respiration in rat liver mitochondria. SKF2314 was active while diethylaminoethanol had no effect. The suggestion was made that the action of these drugs as inhibitors of cholesterol biosynthesis could be partly explained on the basis of a lack of ATP for the biosynthetic pathway. An analogy was drawn to 2,4 - dinitrophenol, both an uncoupler and an inhibitor of sterol biosynthesis.[12]

SKF525-A has a number of other biological effects: inhibition of certain microsomal drug detoxification reactions in mammals,[13] and inhibition of both PMS catalyzed photophosphorylation and the ferricyanide Hill reaction in isolated spinach chloroplasts.[14] The latter work, on the effects of SKF525-A on isolated spinach chloroplasts, gave part of the impetus for the present study. For while it is the diphenylpropylacetate end of the SKF525-A molecule that is the active inhibitory end of the SKF525-A molecule in mitochondrial systems, it is the diethylaminoethyl end of the molecule that is the active inhibitory end in chloroplasts.[14] It was, in part, with this in mind that we investigated some of the details of SKF525-A's effect on rat liver mitochondria. The hope was that not only would the study be pharmacologically interesting in that SKF525-A's

effect on mitochondria would be revealed in some depth, but that the drug's actions might be helpful in illuminating some aspect of mitochondrial energy coupling not as yet fully understood.

Some of the physico-chemical properties of SKF525-A in aqueous solution present difficulties in the interpretation of the experiments reported here. SKF525-A is a surface active molecule, lowering the surface tension of water and forming micelles of varying sizes depending on the salt concentration.[51] Although the critical micelle concentration of SKF525-A is between 10^{-3} and 10^{-2} molar[51] (thus making surface tension considerations of questionable biological significance in general), local, higher concentrations due to absorption and concentration in membranes, and therefore biological effects, cannot be ruled out. SKF525-A has been shown to penetrate cholesterol and lecithin monolayers at concentrations used in this study (2×10^{-6}M).[51]

SKF525-A was examined as a possible uncoupler via the following mitochondrial energy coupled activities: respiration and phosphorylation, calcium ion uptake and hydrogen ion ejection, reverse electron flow, ATPase activity, and the interaction of SKF525-A with the known uncoupler DNP and the known energy transfer inhibitor, oligomycin. Studies with submitochondrial particles, with parts of the SKF525-A molecule and SKF525-A analogs, on SKF525-A binding to mitochondria, and on the

respiratory inhibition caused by high SKF525-A concentrations were auxiliary to the main thesis question (is SKF525-A a true mitochondrial uncoupler and how does it work?) and were not studied in depth. Observations of mitochondrial swelling through spectrophotometry and electron microscopy, although also not studied in depth, provided a major clue toward an interpretation of SKF525-A's action.

METHODS

Preparation of mitochondria. Liver mitochondria were prepared from male, albino, Wistar strain rats (150-200g) by the method of Johnson and Lardy[15] using 0.25M sucrose and 0.01M HEPES buffer, pH7.3 as the isolation medium.

Assay of respiration and oxidative phosphorylation. Mitochondrial respiration was measured at 25°C with a stationary Clark electrode (Yellow Springs Instrument Co.) attached to a Gilson Oxygraph (Gilson Medial Electronics). The reaction medium for liver mitochondria with succinate as substrate[16] contained: (in 3.3 ml final volume): 0.225M sucrose, 0.02M HEPES, pH7.3, 5mM $MgCl_2$, 0.02M KCl, 3.03×10^{-7}M rotenone, and 0.01M potassium phosphate buffer, pH7.3. With glutamate as substrate,[17] the reaction medium contained: (in 3.3 ml final volume): 0.167M sucrose, 0.56mM $MgCl_2$, and 5.6mM potassium phosphate buffer, pH7.3. State 3 and 4 respiratory rates were obtained directly from the records. The respiratory control ratio was calculated as the ratio of the state 3 respiratory rate to the following state 4 respiratory rate. ADP/O ratios were calculated according to Chance and Williams.[18] ADP/O ratios were confirmed with P/O ratios which were obtained using manometry and Sumner's method of phosphate determination.[19] The manometric procedure followed Umbreit[20] and Slater[21] and the medium was the same as for

polarography except for the use of 1mM ATP, 30mM glucose, and an excess of yeast hexokinase (Sigma, type III).

Assay of ATPase activity. The ATPase (E.C.#3.6.1.3) reaction was carried out at 25°C and determined by the estimation of inorganic phosphate.[17] Mitochondria were incubated in a medium containing: (in 1 ml final volume): 0.05M HEPES, pH 7.3, 5mM $MgCl_2$ (when used), and 5mM ATP. The reaction was begun by adding ATP. The reaction was stopped by adding 0.5 ml of 20% TCA, and inorganic phosphate liberated from ATP was assayed by the procedure of Sumner.[19]

Assay of Calcium uptake and hydrogen ion ejection. Calcium uptake was measured by the procedure of Carafoli et al[22] in a medium containing (in 3.3 ml final volume): 10mM HEPES pH 7.3, 80mM NaCl, 10mM succinate, and 200 to 500 mumoles of Ca^{+2} containing about 125,000 CPM $^{45}Ca^{+2}$. The medium contained no phosphate and enough oligomycin to block any state 4 to state 3 transitions. Ca^{+2} was added to a reaction cell (containing mitochondria and all reaction components including drugs) in which oxygen uptake was being monitored polarographically and in which hydrogen ion fluxes were being monitored by a Radiometer combination pH electrode linked through a Radiometer pHM 26 pH meter to a Honeywell Electronik 19 recorder. The recorder was adjusted so that full scale deflection represented 0.1 pH unit.

At the end of the Ca^{+2} induced respiratory stimulation the reaction medium was drawn into a syringe and, using a Swinney adaptor, forced through a millipore filter of 0.45u pore size.[22] A 0.5 ml aliquot of the clear filtrate was analyzed for $^{45}Ca^{+2}$ using a Nuclear Chicago, low-background planchet counter. Ca^{+2}/O ratios were calculated as the uatoms of Ca^{+2} taken into the mitochondria during Ca^{+2}- stimulated respiration, divided by the uatoms of oxygen taken up during Ca^{+2}- stimulated respiration. Microatoms of H^{+} ejected from the mitochondria during the Ca^{+2} - stimulated respiration were determined by comparing the experimental pH (pen) deflection to the pH deflection obtained in identically set up reaction cells when a few mumoles of H^{+} (as HC1) were added to the cells. This procedure circumvented the problem of calculating the buffering capacity of the medium.

Assay of reverse electron flow. Reverse electron flow was measured by the procedure of Azzone et al.[23] in which the disappearance of acetoacetate is monitored in a system using succinate as the source of electrons. The reactions were carried out in Warburg flasks so that (when called for) oxygen uptake could be simultaneously followed manometrically. The reaction medium contained (in 2.5 ml final volume): 50 mM sucrose, 50 mM KC1, 8 mM $MgC1_2$, 20 mM HEPES pH7.3, 10 mM succinate, 5 mM acetoacetate (Sigma), and, when used, 110 ug of antimycin per

gram of mitochondrial protein. The medium contained no inorganic phosphate and enough oligomycin to block state 4 to state 3 transitions. Manometric procedures followed Umbreit et al.[20] and Slater.[21] Flasks were incubated for 20 minutes at 30°C. Reactions were stopped by the addition of 1 ml of 1.5M PCA, centrifuged, and analyzed for acetoacetate by the method of Walker.[24]

Preparation of submitochondrial particles: Submitochondrial particles were isolated by a modification of the procedure of Gregg.[25] The mitochondria from six 500g rats were suspended in the usual isolation media at a concentration of 15 mg of protein per ml and sonicated in 60 ml aliquots as follows: 30 seconds of maximum sonic energy from a Branson sonifier at 0°C followed by 1 minute of cooling (ice bath), repeated until phase microscopy revealed the disappearance of most mitochondria. About 15 cycles were required on most days. The sonicate was centrifuged at 15,000 x g for 5 minutes in a Sorvall RC2-B refrigerated centrifuge to remove whole mitochondria. The supernatant liquid was then centrifuged at 130,000 x g for 60 minutes in the SW25.1 rotor of a Beckman model L ultracentrifuge. The deep red gelatinous pellet was resuspended in the usual isolation medium and used as submitochondrial particles.

Electron microscopy of mitochondria. Actively respiring and

phosphorylating mitochondria were taken by Pasteur pipette from the reaction cell (being monitored for oxygen uptake polarographically in the succinate medium) at various times after drug addition and in two states (completely uncoupled and completely inhibited). Mitochondria were fixed in a mixture of 1 part of 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH.7.4 containing 10 ug/100 ml $CaCl_2$ and 4% sucrose, plus 2 parts of 1% OsO_4 in 0.1 sodium cacodylate buffer, containing 10 ug/100 ml $CaCl_2$ and 4% sucrose, pH 7.4. Fixation time was 1/2 hour at 4°C. After fixation the pellet was stained in 1% aqueous uranyl acetate. Dehydration in an ethanol series was followed by infiltration with propylene oxide. Material was embedded in Epon 812 and dried at 60°C for 48 hours. Sections were cut on an MT-2 Porter-Blum microtome with a diamond knife, mounted on copper grids and stained with 2% uranyl acetate (in 50% alcohol) for 1 minute, followed by 0.4% lead citrate for 5 minutes. Electronmicrographs were made with a Phillips 300 electron microscope.

Partition coefficients. Partition coefficients were calculated between chloroform and water by the method of Skidmore and Whitehouse[26] except that the buffer was HEPES and the medium pH was 5 in order to keep the drugs in solution at the concentrations necessary to obtain reliable optical density measurements on a

Beckman DB spectrophotometer. Drug concentrations were correlated with optical density readings at 258nm.

Mitochondrial swelling. Mitochondrial swelling was observed (not systematically measured), by suspending mitochondria in the succinate assay medium described above and recording O.D. changes at 540 nm in a Beckman DB spectrophotometer when SKF525-A was added to the experimental cuvette.

Protein determination. Protein was determined by the Lowry method[27] using crystalline bovine serum albumin as a standard.

Reagents. Oligomycin, rotenone, antimycin, SKF2314, and diphenylethylacetic acid were dissolved in 95% ethanol. The 95% ethanol was not inhibitory at the levels employed in these experiments. All other compounds were obtained from commercial sources and were dissolved in glass distilled, deionized water.

Source of drugs. SKF525-A and related compounds were obtained through the generosity of the following people: diphenylethylacetic acid, Professor F. Ravenna, Research Laboratories, Maggioni and Co., Milan; SKF525-A, SKF3301-A, SKF16467-A, SKF2314, and triparanol citrate, Dr. W. L. Holmes, Smith, Kline and French Laboratories, Philadelphia; Sch 5706 citrate and Sch 5712, citrate, Professor P. Galimberti, Schelabor, S.p.A., Milan; CFT 1201 and CFT1208, Chemische Fabrik Tempelhof, Preutz and Temmler, Berlin.

All experiments were repeated at least 3 times with essentially identical results and most were done a minimum of 6 times. Variations in values from day to day were within 30% depending on the condition of the isolated mitochondria.

RESULTS

SKF525-A effects on mitochondrial respiration and phosphorylation. A typical effect of SKF525-A on succinate respiration by rat liver mitochondria is shown in figure 1. The respiratory control ratio and ADP/O ratio calculated from figure 1, in the absence of SKF525-A, were 3.5 and 1.4 respectively. The addition of 6.1×10^{-5} M SKF525-A showed no effect on state 3 respiration, but stimulated state 4 respiration to 177% of the control rate. The respiratory control ratio and ADP/O ratio in the presence of 6.1×10^{-5} M SKF525-A were 1.9 and .99 respectively. Table 2 shows the effect of varying SKF525-A concentrations on state 4 and state 3 respiration, on the ADP/O ratio, and on the respiratory control ratio. Figure 2 presents this date in graph form. At lower concentrations, SKF525-A stimulated state 4 respiration and had no effect or slight inhibition (at 9.12×10^{-5} M SKF-525-A) on state 3 respiration. At slightly higher concentrations, SKF525-A inhibited both state 3 and state 4 respiration. The ADP/O ratio and respiratory control decreased and eventually reached zero (ADP/O) and 1 (respiratory control ratio) as the SKF525-A concentration increased.

Table 3 presents similar date for mitochondrial respiration in state 3 and phosphate uptake using manometry and an analytical method of phosphate determination (Sumner's method). The results by these methods are essentially the same as obtained

by polarography. Table 4 compares the action of SKF525-A on respiration and phosphorylation when either succinate or the NAD^+ linked substrate, glutamate, is used. The pattern of effect on glutamate respiration and phosphorylation is seen to be similar to the effects with succinate as substrate. Figure 3 shows the interaction of SKF525-A with oligomycin. When oligomycin is present in concentrations sufficient to prevent state 4 to state 3 transitions, SKF525-A is able to release part of this inhibition.

SKF525-A effects on mitochondrial uptake of Ca^{++} and ejection of H^+: Table 5 shows the effect of increasing SKF525-A concentration on the uptake of calcium by mitochondria. As the SKF525-A concentration increased, the amount of calcium taken up by the mitochondria decreased and eventually reached an inhibition of 92% compared with the control. At the same time, the amount of H^+ released into the medium by the mitochondria, as a result of the calcium uptake, decreased and eventually reached zero as the SKF525-A concentration increased. During the period of calcium uptake, with increasing SKF525-A concentrations, oxygen uptake increased and only dropped off (still above control levels), at the highest concentration of drug. The stimulation of calcium uptake seen at 3.03×10^{-6}M SKF525-A was a consistent feature of the SKF525-A activity.

SKF525-A effects on mitochondrial reverse electron flow:

Table 6 illustrates the effect of increasing SKF525-A concentrations on the disappearance of acetoacetate from a system where succinate can feed electrons both to oxygen and, in a reverse pathway, to NAD. At lower SKF525-A concentrations, the amount of acetoacetate reduced gradually decreased and the amount of oxygen reduced increased. At higher SKF525-A concentrations (6×10^{-5} M and greater), both acetoacetate reduction and oxygen reduction decreased, the inhibitory levels being reached abruptly. The use of rotenone and DNP decreased the acetoacetate reduction and, in the case of DNP, increased the uptake of oxygen.

SKF525-A effects on mitochondrial ATPase activity: table 7 shows the effect of SKF525-A on mitochondrial ATPase activity with and without added Mg^{++}. With Mg^{++} present, ATPase activity was stimulated to a maximum at 3×10^{-4} M SKF525-A and then decreased slightly from this value. Without the addition of Mg^{++}, 1×10^{-4} M SKF525-A elevated the ATPase activity from a control rate to a value roughly comparable to the system with Mg^{++} present (and an SKF525-A concentration of 1×10^{-4} M). But further increases in the SKF525-A concentration lowered the ATPase activity from the high point but still kept it above control levels. Concentrations of SKF525-A lower than those employed in the above experiments had no effect on the ATPase activity. Higher con-

centrations of SKF525-A precipitated out of solution.

SKF525-A interaction with DNP in the mitochondrial ATPase reaction: table 8 repeats the experiment shown in table 7 with the addition to all reaction vessels of 1×10^{-4} M DNP. In the presence of Mg^{++}, increasing SKF525-A concentrations lowered the already stimulated rate by about 25% (at 5×10^{-4} M SKF525-A). In the absence of added Mg^{++}, 1×10^{-4} M SKF525-A was able to further stimulate the system, but further increases in the SKF 525-A concentration inhibited the system below the control level. But in both systems (with and without Mg^{++}) containing DNP, the lowest ATPase levels were always above control levels obtained when DNP was absent (table 7).

SKF525-A interaction with oligomycin in the mitochondrial ATPase reaction: The ability of oligomycin to inhibit SKF525-A stimulated ATPase activity in the presence of Mg^{++} is shown in table 9. 3×10^{-4} M SKF525-A by itself and 1ug/ml oligomycin by itself changed the ATPase activity from control levels to 188% and 50% of the control rate respectively. Mixed together, the ATPase activity was 77% of the control rate.

SKF525-A interaction with sonically produced submitochondrial particles. Figure 4 illustrates the pattern of respiratory inhibition of submitochondrial particles (respiring in the presence of ADP and P_i) when the SKF525-A concentration varied from

1×10^{-6} M to 2×10^{-4} M. The lack of stimulation seen in whole mitochondria respiring in the presence of ADP and P_i (table 2) is also observed in submitochondrial particles. The respiratory inhibition seen at higher SKF525-A concentrations in whole mitochondria (tables 2 and 3) is also present with submitochondrial particles.

Comparison of the effects of SKF525-A with the two parts of the SKF525-A molecule and analogs of SKF525-A. Table 10 compares the ability of SKF525-A, parts of the SKF525-A molecule, and analogs of SKF525-A, to raise the state 4 respiratory rate to 150% of the control level and to lower the P/O ratio to 50% of the control level. SKF525-A was seen to be the most active compound in inhibiting phosphorylation; the diphenyl-propylacetate end of SKF525-A, called SKF2314, was within one order of magnitude less active than SKF525-A. DPEAA was about as effective as SKF2314 indicating that the propyl group of SKF2314 was not uniquely essential for its activity (see table 1 for structures). Diethylaminoethanol, the other part of the SKF525-A molecule, had no effect on the mitochondrial reactions considered in this study. An oxygen bridge between the 2 parts of the molecule (as seen in SKF3301-A) seemed to be more effective than a nitrogen bridge (as seen in SKF16467-A) in conferring activity to the molecule. The presence of a nitrogen

bridge in Sch5706 may also be responsible for the lack of effectiveness of this molecule. The presence of longer carbon chain subtituents on the diphenylpropylacetate part of the SKF525-A molecule seemed to lead to increased activity (compare CFT1201 and CFT1208, and Sch 5706 and Sch 5712).

Partition coefficients of SKF525-A, the two parts of the SKF 525-A molecule, and certain analogs. Table 11 shows that the order of decreasing ability to partition mainly in the nonpolar solvent (chloroform) was: SKF2314, SKF525-A, SKF3301-A, SKF 16467-A, Sch 5706, and DEAE. A comparison of table 11 with table 10 indicates that, with the exception of SKF2314, an increasing ability to partition in the nonpolar solvent correlated with an increasing activity of the molecule. The validity of this correlation is somewhat questionable, however, due to the methodology (explained above) requiring measurement of partition coefficients at pH5 and not at pH 7.3, the pH of the other experimental procedures.

Electron microscopy of control and SKF525-A treated mitochondria. When mitochondria were treated with SKF525-A they showed severe swelling and mild clumping (plates 1 - 4). This swelling was also observable as 1) an obvious visual clarification of the mitochondrial suspension when SKF525-A was added to it and 2) optical density decreases of the order of 0.3 OD units in the mitochondrial suspension when SKF525-A was added to it.

Binding of SKF525-A to mitochondria. Figure 5 illustrates the possible pattern of binding of SKF525-A to mitochondria. Mitochondria were titrated against a constant drug concentration.[28] If the initial dosage of mitochondria bound the drug tightly, then succeeding doses of mitochondria should not have been as severely affected by the drug-mitochondrial suspension. This should have led to a non-straight line relationship between mitochondrial concentration and respiratory rate, and the curves showing mitochondrial respiration in the presence of uncoupling concentrations of SKF525-A should have gradually fallen from a straight line and approached the control curve as the mitochondrial concentration increased. This however does not occur and there seems to be a straight line relationship, with and without drug, between respiration and mitochondrial concentration. This suggests that within the range of mitochondrial concentrations tested, the effect of SKF525-A was a function of the drug final concentration in the medium,[28] and not a stoichiometric relationship.

Localization of the electron transport block by higher concentrations of SKF525-A. Figure 6 shows that the inhibition to electron flow brought about during succinate respiration by higher concentrations of SKF525-A, could be overcome by the use of the ascorbate-TMPD electron feeder system. This presumably places the block to electron flow on the substrate side of cytochrome c. However, other interpretations of this data are considered in the discussion.

DISCUSSION

Respiratory inhibitors may be classified into 3 types: electron transfer inhibitors, uncouplers, and energy transfer inhibitors.[29] Whitehouse considered SKF525-A to be an uncoupler of oxidative phosphorylation on the basis of lowered P/O ratios in drug treated mitochondria as compared with controls.[12] A mitochondrial uncoupler, in general, dissipates energy that could have been used to power ion movements, volume and structural changes, transhydrogenation reactions, and reverse electron flow, as well as phosphorylation reactions. SKF525-A's uncoupling activity has been investigated under some of these criteria in an extension of Whitehouse's findings.

The manifestations of uncoupling as measured by the reactions just mentioned are not proof that a molecule is a true mitochondrial uncoupler interacting specifically with some high energy intermediate in the phosphorylating mechanism. In fact, most of the data describing the activity of SKF525-A are best explained through a non-specific, detergent-like activity of the molecule on the mitochondrial membranes. This non specific action due to the surfactant properties of the molecule may account for the "uncoupling" data.

SKF525-A inhibited ATP production while allowing electron flow to proceed at control levels or at stimulated rates (figures 1 and 2, tables 2 to 4; the respiratory control ratios obtained consistently in these experiments indicated a lack of very tight coupling of the mitochondrial preparations. Respiratory control coefficients of

4 for succinate and 4 for glutamate are too low to indicate truly tight coupling and may have been due to impurities in our water--deionized and then glass distilled--or other factors we were not able to control). Calcium uptake and hydrogen ejection (table 5), and reverse electron flow (table 6) were also inhibited at concentration allowing electron flow to proceed at control or stimulated rates. In addition, as is characteristic of uncouplers,[32,33] ATPase activity was stimulated (table 7), oligomycin induced inhibition of state 3 respiration was relieved in the presence of SKF525-A (figure 3), and oligomycin inhibited SKF525-A stimulated ATPase activity (table 9). The interaction of SKF525-A with mitochondria is about as rapid as the interaction of ADP with mitochondria as seen in figures 1, 3, and 6. The interaction of SKF525-A with mitochondria is therefore virtually instantaneous and does not change with time. Table 12 presents the concentration of known uncouplers and SKF525-A necessary for inducing maximal stimulation of state 4 respiration (with concomitant loss in phosphorylation) and for inducing 50% inhibition of phosphorylation. The concentration of SKF525-A necessary for maximal stimulation of state 4 respiration is within the range of concentrations shown by the known mitochondrial uncouplers (table 12). SKF525-A was capable of completely abolishing phosphorylation (or nearly so), while electron flow continued uninhibited (tables 2 and 3). The known uncouplers act in the same way but possibly over a greater range

of concentrations.[40,41] Inhibition of phosphorylation and reverse electron flow as well as inhibition of foreward electron flow (after phosphorylation or reverse electron flow had been completely inhibited) set in rapidly with small increments in drug concentration (tables 2, 3, and 6).

This rapid onset of inhibition is similar to the curve obtained when antimycin is titrated against respiring mitochondria and has been interpreted as the binding of the drug to specific sites in the mitochondrial respiratory chains.[42] The binding does not produce inhibition until a critical concentration is reached supposedly due to the ability of electrons to be transferred between adjacent respiratory chains until all or the majority of the chains have been inhibited (bound with drug).[42]

SKF525-A affected calcium uptake in such a way that this system could be essentially completely inhibited with no loss in the rate of electron flow (table 5). SKF525-A was also able to discharge calcium from mitochondria previously loaded with calcium in the absence of SKF525-A. SKF525-A appeared to act like DNP with respect to the inhibition of calcium uptake. Both compounds dissipated the energy needed to power this process within minutes (that is, it took a few minutes for the discharge of all the calcium previously loaded into the mitochondria), and both compounds acted maximally at roughly the same concentration: 1×10^{-4}M DNP[35] and 2.5×10^{-4}M SKF525-A are approximately equivalent. Dicoumarol,

m-Cl-CCP, and gramicidin are more potent acting at 10^{-5}M, [36] 10^{-7}M, [36] and 10^{-9}M, [36] respectively. Arsenate is less potent acting at 10^{-3}M.[37] The mitochondrial system reported here differed from most published systems in that our efficiency of control calcium uptake was lower than theirs. Up to 99% of the calcium in the medium has been reported to be accumulated by mitochondria.[38] Our systems consistently accumulated about 40% of the medium calcium. It is questionable therefore whether or not our system ever accumulated calcium against a concentration gradient. However the behavior of the system with respect to bursts of oxygen uptake on the addition of calcium,[38] the inhibitory action of DNP on our system,[38] and the discharge of calcium from the mitochondria induced by anaerobiosis[38] led us to conclude that the uptake of calcium was energy linked. The action of oligomycin also led us to believe that the system was valid: while both ADP or Ca^{+2} addition to respiring mitochondria led to bursts of respiration followed by a return to the previous, slower rate, the presence of oligomycin had the effect of abolishing the ADP stimulation without affecting the Ca^{+2} stimulation. This effect, illustrated in figure 8, places the site of interaction of Ca^{+2} and the phosphorylating chain at a point earlier in the chain than the site of interaction of the chain with either ADP or oligomycin. This effect is in line with current thinking which considers the non-phosphorylated high energy state of the mitochondrion as the source of energy for calcium ion

movements.[39]

SKF525-A blocked reverse electron flow completely at 10^{-4}M while not inhibiting foreward electron flow (table 6). It is slightly less active than DNP and dicoumarol; the latter two inhibit reverse electron flow at 5×10^{-5}M[23] and 2×10^{-5}M[23] respectively. Essentially identical results were obtained in antimycin inhibited mitochondrial systems, thus insuring that succinate was the sole donor of electrons for reverse electron flow (table 13). Concentrations of SKF525-A that inhibited acetoacetate reduction in this system (1×10^{-4}M and greater) failed to inhibit succinate dehydrogenase (table 6, 1×10^{-4}M SKF525-A) and B-hydroxybutyrate dehydrogenase (as measured in whole mitochondria polarographically, table 14). These data eliminated the 2 dehydrogenases involved in the assay as sites of inhibition and seemed to place the site of action of SKF525-A within the energy coupling chain. Since SKF525-A was effective in systems inhibited with oligomycin and lacking exogenous phosphate, within the scope of this experiment the non-phosphorylated high energy state of the mitochondria was implicated as the site of action of SKF525-A. The effectiveness of SKF525-A on the calcium uptake system under similar conditions (presence of oligomycin and no exogenous phosphate) also implicated non-phosphorylated high energy state of the mitochondrion. However, this is in no way a proof that the non-phosphorylated high energy state is the specific site of action of SKF525-A since the mitochondrial phosphorylating

mechanism is known to be more labile than the respiratory chain to physical disruption, whether by mechanical means or via detergents. Similarly, the stimulatory effect of SKF525-A on mitochondrial respiration in the absence of both phosphate acceptor and phosphate implicated but did not prove the non-phosphorylated high energy state as the site of action of SKF525-A.

SKF525-A stimulated ATPase activity with or without added Mg^{++} (table 7). ATPase activity reached a maximum and then fell off, but remained above control levels. Greater stimulation was possible if exogenous Mg^{++} was present. DNP yielded similarly shaped curves in our experiments and in other work.[40,41] However, SKF525-A required higher concentrations for maximal ATPase stimulation than did DNP and a number of other uncouplers. Concentrations for maximal ATPase stimulation by DNP, dicoumarol, m-Cl-CCP and chlorpromazine were, respectively, 2×10^{-6}M, [40] 10^{-5}M, [43] 2.5×10^{-6}M, [31] and 2.1×10^{-5}M.[28] In the absence of Mg^{+2} SKF525-A induced maximal ATPase activity at 1×10^{-4}M (table 7).

The interaction of SKF525-A and DNP (table 8) may be explained as an additive drug effect. That is, although the % control ATPase activity fell from 100% to 27% (in the case of the curve with no exogenous Mg^{+2} present), the actual activity fell from 2.49 to 0.67 (umoles/mg)/10 min, the latter value reasonably being a point on either a DNP or SKF525-A curve that employed the drugs at a concentration equal to the sum of the concentrations of both DNP

and SKF525-A.[40,41] The same argument can be given for the SKF525-A and DNP interaction curve with added Mg^{+2}.

After observing the effect of SKF525-A on whole mitochondria, we thought it would be interesting to test the effect of the drug on respiration in sonically produced submitochondrial particles.

It was hoped that the partial destruction of the mitochondrial membranes achieved by sonically producing submitochondrial particles would modify or eliminate that membrane component (s) and thus lead to an altered, interpretable response of the mitochondria to SKF525-A (as compared with whole mitochondria). The sonically produced submitochondrial particles had the property of "reverse acceptor control",[44] that is, ADP inhibited respiration as opposed to its effect on whole, coupled mitochondria. P_i orP_i plus ADP stimulated respiration.[44] Concentrations of SKF525-A that stimulated respiration in whole, coupled mitochondria had no effect on the respiratory rate of submitochondrial particles (respiring in the presence of phosphate and ADP). Concentrations of drug that inhibited respiration in whole coupled mitochondria also inhibited respiration in submitochondrial particles. Since the sonically produced submitochondrial particles lacked respiratory control it was expected that SKF525-A would not be able to stimulate respiration. The inhibition at higher concentrations may have been due to the solubilizing of the mitochondrial membranes.

As expected from previous work,[41] the more lipophilic the SKF525-A analog was, the more active it was (tables 10 and 11;

the method for calculating the partition coefficient gives large changes in the coefficient with very small OD changes. Therefore, the partition coefficients varied by as much as 40% from day to day but the relative solubilities remained the same). One exception was SKF2314, part of the SKF525-A molecule, which was the most lipophilic of the compounds tested (table 11) but the eighth most active in stimulating respiration and inhibiting phosphorylation. This may indicate that SKF2314 either has a10 fold lower affinity than SKF525-A for the binding site (s) leading to drug activity (table 10), or due to its difference in charge (SKF2314 has a dissociable hydrogen where as SKF525-A can pick up a hydrogen ion on the terminal nitrogen atom) it acts on a different site than SKF525-A.

Electron microscopy revealed mild clumping and severe swelling of the SKF525-A treated mitochondria (plates 1-4). This is opposed to the effect of DNP and mC1-CCP which inhibit swelling.[47] Volume changes could also be observed spectrophotometrically as optical density decreases in the range of 0.3 OD unit on addition of SKF525-A to the mitochondria. The prevention rather than the promotion of (energy linked)swelling was an expected property of SKF525-A since the molecule exhibited so many properties of an uncoupler.[47] That swelling did occur and was not the typical large amplitude swelling (the mitochondria under the influence of SKF525-A did not increase in overall size but seemed to have a decrease in the amount of

stainable cristae; it was as if the matrix space were expanding) raised the possibility that the cristae were being dissolved by the SKF525-A molecule due to its surface active properties. This interpretation would mean that the "uncoupling" reactions seen in the various assays undertaken in this study were secondary effects of the dissolution of the inner mitochondrial membrane. It may be important to study mitochondrial volume changes systematically as a function of concentration since the electron micrographs (plates 1-4) were taken at SKF525-A concentrations that completely uncoupled the mitochondria. It is possible that lower concentrations may not induce the "swelling" or "dissolving" seen at higher concentrations. Electron micrographs of mitochondria incubated with drug concentrations ten times higher than the concentrations used in plates 2-4 looked just like the lower drug concentration plates.

The simplest explanation for the inhibition of mitochondrial respiration by higher concentrations of SKF525-A (concentrations beyond stimulating concentrations), is that at higher concentration, the mitochondrial membranes are being dissolved by the drug. While Van Dam and Slater have proposed models for the inhibition of mitochondrial respiration at high uncoupler concentrations,[30,48,49,50] the present data for SKF525-A's action does not warrant a consideration of their mechanism.

It is difficult to conclude whether surface activity is incidental to drug action (the result of the detergent like structure of the SKF525-A

molecule) or the reason for drug function. The inhibition of various enzymes could be due to specific binding of the drug to the membrane components involved or due to a non-specific binding and solubilizing of the mitochondrial membranes in general.

SKF525-A seemed to be a mitochondrial uncoupler acting on the nonphosphorylated high energy state of the mitochondrion in a manner similar to that of DNP. This conclusion was based on the action of SKF525-A on phosphorylation, calcium ion uptake, reverse electron flow, ATPase activity, and interactions and comparisons with known uncouplers, DNP in particular, and the energy transfer inhibitor oligomycin. Data on the lipid solubilizing of the molecules on the inhibition of respiration at high drug concentration and the interaction of SKF525-A with submitochondrial particles were consistent with a chemical interpretation. However, studies with the election microscope and observations of mitochondrial swelling have suggested the uncoupling activity to be secondary to nonspecific structural alterations of the mitochondrial membranes. This explanation can, at present, most simply explain the activity of the drugs: at low concentration of drug the phosphorylating mechanism is disrupted through solubilizing of the mitochondrial inner membrane and the apparent uncoupling activities are seen; at higher drug concentrations the respiratory chain is inhibited (through gross disruption of the inner membrane structure) and thus again mimics the effect (respiratory inhibition) of a true uncoupler at higher

concentration.

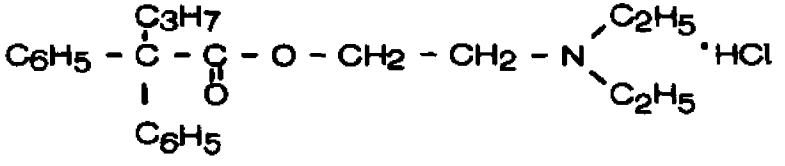
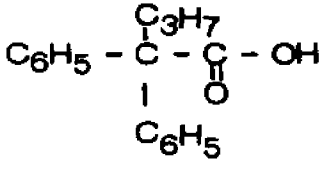
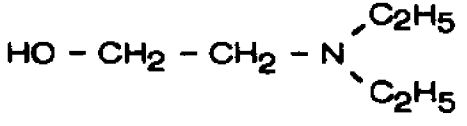
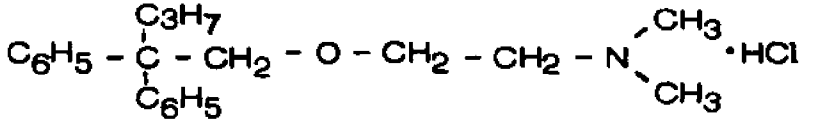
Drug binding experiments suggested a non stoichiometric relationship between drug concentration and degree of effect on mitochondrial respiration. This is opposed to the observations of rapid onset of SKF525-A action (p. 18) which suggested a specific binding to mitochondrial components. It may be that the binding experiments utilized too high a concentration of SKF525-A or too low a concentration of mitochondria to distinguish the true pattern of binding. Greater clarification is needed on this point and on the nature of the physical chemical interaction of SKF525-A with mitochondrial membranes.

That SKF525-A did bring about "uncoupling" reactions may be an indication of the way uncouplers work even though the action of the drug may be "nonspecific". Both DNP and SKF525-A inhibit oxidative phosphorylation and simultaneously stimulate state 4 respiration at lower concentrations than required to induce ATPase activity; this may indicate a disorientation of structural components of the membrane rather than hydrolysis of labile intermediates as the mode of action of the compounds. This mode of action may be consistent with the ability of SKF525-A to disrupt membranes and may, by analogy, lend support to this mode of action for DNP.

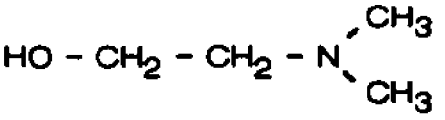
The effect of SKF525-A on the mitochondria membranes may warrant further investigation since the electron micrographs indicate that at the employed concentrations of SKF525-A the inner membrane is "dissolving" while the outer membrane appears intact. This diff-

erential activity may be due to the differing lipid compositions of the two membranes (the outer membrane being higher in cholesterol concentration and lower in cardiolipin concentration than the inner membrane[52]) and may be valuable as a way of separating the 2 membranes from each other.

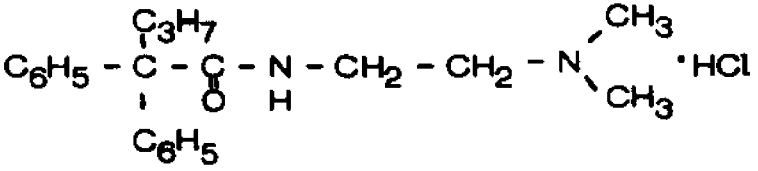
Table 1: Names and structural formulas of the compounds used in this study.

Compound	Structure
1. B-diethylaminoethyl-diphenylpropyl acetate •HCl (SKF525-A)	
2. Diphenylpropylacetic acid (SKF2314)	
3. Diethylaminoethanol (DEAE)	
4. 2,2 - Diphenyl - 1 - (B-dimethylaminoethoxy) pentane • HCl (SKF3301-A)	

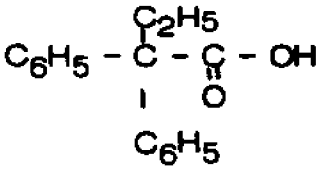
5. Dimethylaminoethanol (DMAE)



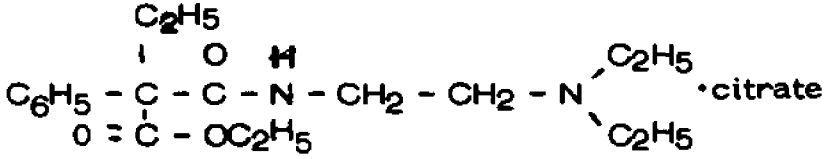
6. B-dimethylaminoethylamino-diphenylpropyl acetate ·HCl (SKF 16467-A)



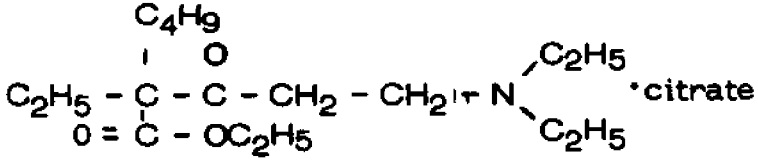
7. Diphenylethylacetic acid



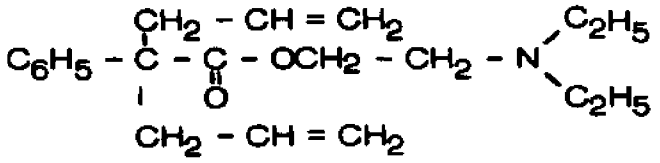
8. Diethylaminoethylamino-phenylethylcarbethoxy acetate·citrate (Sch 5706·citrate)



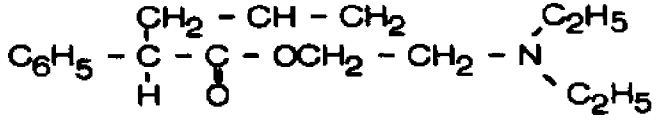
9. Diethylaminoethylbutylethylcarboethoxy acetate ·citrate (Sch 5712·citrate)



10. Diethylaminoethyl-diallylphenyl acetate (CFT 1201)



11. Diethylaminoethyl-allylphenyl acetate (CFT 1208)



12. 1 - (p-(Bdiethylaminoethoxy) phenyl) - 1 - (p-tolyl)- 2 - p-chlorophenyl ethanol ·citrate (Triparanol·citrate)

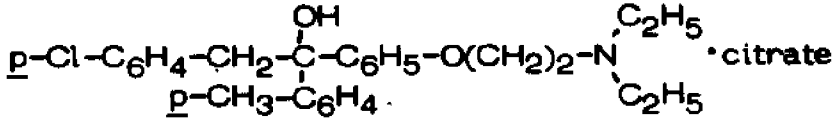


Table 2: The effect of varying concentrations of SKF525-A on respiration in states 3 and 4, on the ADP/O ratio, and on respiratory control in isolated rat liver mitochondria.[a]

SKF525-A Conc. (molar)	Repiratory Rate (muatoms/min)/mg pr				ADP/O	Respiratory Control
	State 4	% Control	State 3	% Control		
0	23.0	100	81.0	100	1.50	3.52
9.0×10^{-7}	23.0	100	81.0	100	1.50	3.52
3.03×10^{-6}	27.2	118	81.0	100	1.50	2.85
3.03×10^{-5}	35.0	152	81.0	100	1.38	2.46
9.12×10^{-5}	38.0	165	59.2	73	0.82	1.55
1.52×10^{-4}	25.6	111	37.2	46	0.0	1.0
3.03×10^{-4}	20.2	88	30.0	37	0.0	1.0
9.12×10^{-4}	6.0	26	6.5	8	0.0	1.0

[a]Experimental conditions described in Methods. The reaction medium contained 0.225M sucrose, 0.02M HEPES, pH 7.3, 5mM $MgCl_2$, 0.02M KCl, 1.8mM succinate, 3.03×10^{-7}M rotenone, and 0.01M potassium phosphate buffer pH 7.3 in a 3.3 ml final volume polarographic cell. 1.98 mg mitochondrial pr/ml.

Table 3: The effect of varying concentrations of SKF525-A on respiration in state 3 and on the P/O ratio in isolated rat liver mitochondria.[a]

SKF525-A Conc. (Molar)	Oxygen uptake (uatoms)	Phosphate uptake (umoles)	P/O ratio
0	8.85	15.3	1.73
4×10^{-6}	9.60	14.1	1.47
6×10^{-5}	9.50	10.2	1.07
9×10^{-5}	8.90	3.4	.38
1.2×10^{-4}	4.25	1.2	.28
2.0×10^{-4}	4.10	1.0	.24
3.2×10^{-4}	0.0	0.0	----

[a]The reaction medium contained 0.225M sucrose, 0.02M HEPES, pH 7.3, 5mM Mg Cl_2, 0.02M KCl, 3.03×10^{-7} M rotenone, 1.8mM succinate, 0.01M potassium phosphate buffer, pH 7.3, 1mM ATP, 30mM glucose, and an excess of yeast hexokinase in a 2.5 ml final volume. 20 minute reaction time. 1.33 mg mitochondrial protein/ml.

Table 4: Comparison of respiration with succinate and the NAD^+ linked substrate glutamate with respect to state 3 and state 4 oxygen uptake, respiratory control, and the P/O ratio.[a]

Substrate	SKF525-A Conc. (M)	Oxygen uptake mumoles/min/mg		% Control		Respiratory Control	ADP/O Ratio
		State 4	State 3	State 4	State 3		
Succinate	0.0	7.74	27.3	177	100	3.5	1.4
	6.1×10^{-5}	13.70	27.3			1.9	0.99
Glutamate	0.0	5.83	21.9	204	100	3.8	2.5
	6.1×10^{-5}	11.90	21.9			1.8	1.9

[a]The succinate and glutamate concentrations were 1.8 and 5.6 millimolar, respectively. The mitochondrial protein concentration with succinate and glutamate was 2.19 and 2.97 mg protein/ml respectively. The glutamate assay medium contained 0.167M sucrose, 0.56M $MgCl_2$, and 5.6mM potassium phosphate buffer, pH 7.3 in a 3.3 ml final volume. Succinate assay medium as in table 2.

Table 5: The effect of SKF525-A on calcium ion uptake, hydrogen ion ejection, and oxygen uptake in isolated rat liver mitochondria.[a]

SKF525-A Conc. (Molar)	Ca^{+2} entering the mitochondria (mumoles)	Oxygen uptake (muatoms)	Ca^{+2}/O Ratio	H^{+} released from the mitochondria (mumoles)	Ca^{+2}/H^{+} Ratio
0	76	48	1.58	72	1.06
3.03×10^{-6}	99	62	1.60	67	1.48
3.03×10^{-5}	78	119	.65	53	1.47
6.06×10^{-5}	50	125	.40	47	1.06
1.21×10^{-4}	24	141	.17	35	.69
2.42×10^{-4}	7	88	.08	0	----

[a]The reaction medium contained (3.3 ml final volume) 10mM HEPES, pH 7.3, 80mM NaCl, 10mM succinate, and 200 mumoles Ca^{+2} containing about 125,000 CPM $^{45}Ca^{+2}$. The medium contained no phosphate and sufficient oligomycin to block state 4 to state 3 transitions. 4.44 mg mitochondrial protein.

Table 6: The effect of SKF525-A on energy linked reverse electron flow in isolated rat liver mitochondria.[a]

SKF525-A Conc. (Molar)	Acetoacetate dissapearance (mumoles)	O_2 uptake during acetoacetate reduction (muatoms/min)/mg
0	19.1	22.8
4×10^{-6}	17.4	24.0
2×10^{-5}	15.3	32.2
4×10^{-5}	14.3	36.2
6×10^{-5}	12.4	28.4
1×10^{-4}	0.0	22.8
2×10^{-4}	0.0	4.2
1.2×10^{-6}M rotenone	7.1	21.8
2×10^{-5}M DNP	13.3	33.0

[a]The reaction medium contained 50mM sucrose, 50mM KCl, 8mM $MgCl_2$, 20mM HEPES, pH 7.3, 10mM succinate, and 5mM acetoacetate in a 2.5 ml final volume. Oxygen uptake monitored manometrically. 5.0 mg mitochondrial protein.

Table 7: The effect of SKF525-A on the ATPase activity of isolated rat liver mitochondria.[a]

SKF525-A Conc. (Molar)	ATPase activity (umoles P_i released/mg) 10 min $+Mg^{+2}$	% Control	$-Mg^{+2}$	% Control
0	0.585	100	0.302	100
1×10^{-4}	0.696	119	0.679	225
3×10^{-4}	2.830	484	0.380	125
5×10^{-4}	2.160	369	0.380	125

[a]The reaction medium contained 0.05M HEPES, pH 7.3, 5mM Mg Cl_2 (when used), and 5mM ATP in a 1 ml final volume. ATP addition began the reaction. Reaction was stopped by adding 0.5 ml 20% TCA. Phosphate was assayed by the procedure of Sumner.[19] 1.70 mg mitochondrial protein.

Table 8: Interaction of SKF525-A with DNP in the ATPase reaction in isolated rat liver mitochondria.[a]

System	ATPase Activity (umoles P_i released/mg) 10 min + Mg^{+2}	% Control	- Mg^{+2}	% Control
1×10^{-4}M DNP	2.72	100	2.49	100
1×10^{-4}M DNP + 1×10^{-4}M SKF525-A	2.74	101	3.31	133
1×10^{-4}M DNP + 2×10^{-4}M SKF525-A	----	----	2.02	81
1×10^{-4}M DNP + 3×10^{-4}M SKF525-A	2.39	88	.77	31
1×10^{-4}M DNP + 5×10^{-4}M SKF525-A	2.01	74	.67	27

[a]Conditions as in Table 7. 1.40 mg mitochondrial protein.

Table 9: Interaction of SKF525-A with oligomycin in the ATPase reaction of isolated rat liver mitochondria.[a]

System	% Control ATPase Activity	ATPase Activity (umole/mg)/10 min
No drugs (+ Mg^{+2})	100	1.45
3 x 10^{-4}M SKF525-A	188	2.73
3.3 ug oligomycin	52	0.75
3 x 10^{-4}M SKF525-A + 3.3 ug oligomycin	77	1.12

[a]Conditions of incubation described in Table 7. 1.43 mg mitochondrial protein.

Table 10: Comparison of the effects of SKF525-A with the two parts of the SKF525-A molecule and analogs of SKF525-A with respect to state 4 respiration and oxidative phosphorylation.[a]

Compound	Concentration Stimulating State 4 Respiration to 150% of Control (mM)	The Concentration Inhibiting the ADP/O Ratio to 50% of Control (mM)
1. CFT 1201	0.016	0.099
2. SKF525-A	0.023	0.098
3. SKF3301-A	0.039	0.156
4. Triparanol · citrate	0.040	0.121
5. Sch 5712· citrate	0.040	0.842
6. CFT 1208	0.090	0.277
7. DPEAA	0.129	0.296
8. SKF2314	0.159	0.311
9. SKF16467-A	0.474	2.895
10. Sch 5706· citrate	2.033	3.881
11. DEAE	No effect	No effect
12. DMAE	No effect	No effect

[a]Experimental conditions as in figure 1. Values given above are the average of 6 - 7 experiments. The pattern of respiratory stimulation and inhibition of phosphorylation (respiration control method) was like that for SKF525-A (see table 2 and figure 1).

Table 11: Partition coefficients of SKF525-A, the 2 parts of SKF525-A, and several analogs of SKF525-A, between chloroform and water.[a]

Compound	Initial OD	Residual OD	Partition Coefficient $\left(\frac{\text{Initial OD}-\text{Residual OD}}{\text{Residual OD}}\right)$
SKF2314	.605	- .005	Infinite
SKF525-A	.492	+ .004	117.0
SKF3301-A	.479	+ .006	81.0
SKF16467-A	.421	+ .010	41.0
Sch5706 · citrate	.251	+ .078	2.2
DEAE	.761	+ .304	1.5

[a]Experimental conditions described in methods. Partition coefficients calculated via the following formula: $\frac{\text{Initial OD - Residual OD}}{\text{Residual OD}}$. All compounds were at an initial aqueous concentration of 1×10^{-3}M. except DEAE which was at an initial aqueous concentration of 1×10^{-1}M.

Table 12: Comparison of SKF525-A with known uncouplers with respect to literature values for both stimulation of state 4 respiration and the loss of phosphorylation in isolated rat liver mitochondria.

Compound	Concentration for maximal stimulation of State 4 Respiration (Molar)	Concentration for 50% inhibition of Phosphorylation (Molar)
1. Valinomycin[34]	1×10^{-8} to 1×10^{-7}	1.4×10^{-8}
2. FCCP[30]	3×10^{-7}	---
3. M - Cl - CCP[31]	4×10^{-7}	1.6×10^{-7}
4. Gramicidin[30]	4.1×10^{-7}	---
5. TTFB[30]	7×10^{-7}	---
6. Dicoumarol[30]	5×10^{-6}	2.0×10^{-6}
7. SKF525-A	2.7×10^{-5}	9.0×10^{-5}
8. Chlorpromazine[28]	1×10^{-4}	1.1×10^{-4}
9. 2,4 - dinitrophenol[30]	3×10^{-4}	2.3×10^{-5}
10. Arsenate[33]	4×10^{-2}	---

Table 13: The effect of SKF525-A on energy linked reverse electron flow in antimycin-inhibited rat liver mitochondria.[a]

SKF525-A Conc. (Molar)	Acetoacetate converted by the mitochondria (mumoles)
0	25.2
4×10^{-6}	21.3
2×10^{-5}	17.9
4×10^{-5}	16.9
6×10^{-5}	14.1
1×10^{-4}	3.2
2×10^{-4}	0

[a]Conditions of incubation described in Table 6 with the presence of about 110 ug antimycin per gram of mitochondrial protein in each reaction vessel. 6.2 mg mitochondrial protein present.

Table 14: The effect of varying concentrations of SKF525-A on state 4 respiration in rat liver mitochondria respiring on B-hydroxybutyrate.[a]

SKF525-A Conc. (molar)	Respiratory Rate (muatoms O/min)/mg protein
0	19.0
9.0×10^{-7}	19.0
3.03×10^{-6}	23.0
3.03×10^{-5}	28.0
9.12×10^{-5}	29.0
1.52×10^{-4}	24.0
3.03×10^{-4}	19.4
9.12×10^{-4}	4.0

[a]B-hydroxybutyrate concentration was 5.6 millimolar. The mitochondrial protein concentration was 3.01 mg/ml. Oxygen uptake recorded polarographically.

FIGURE 1

Respiratory pattern of liver mitochondria. The oxygen uptake was measured polarographically in the succinate medium described in Methods. Rate of oxygen uptake expressed as (muatoms/min)/mg protein, and shown to the left of the slopes on the record.

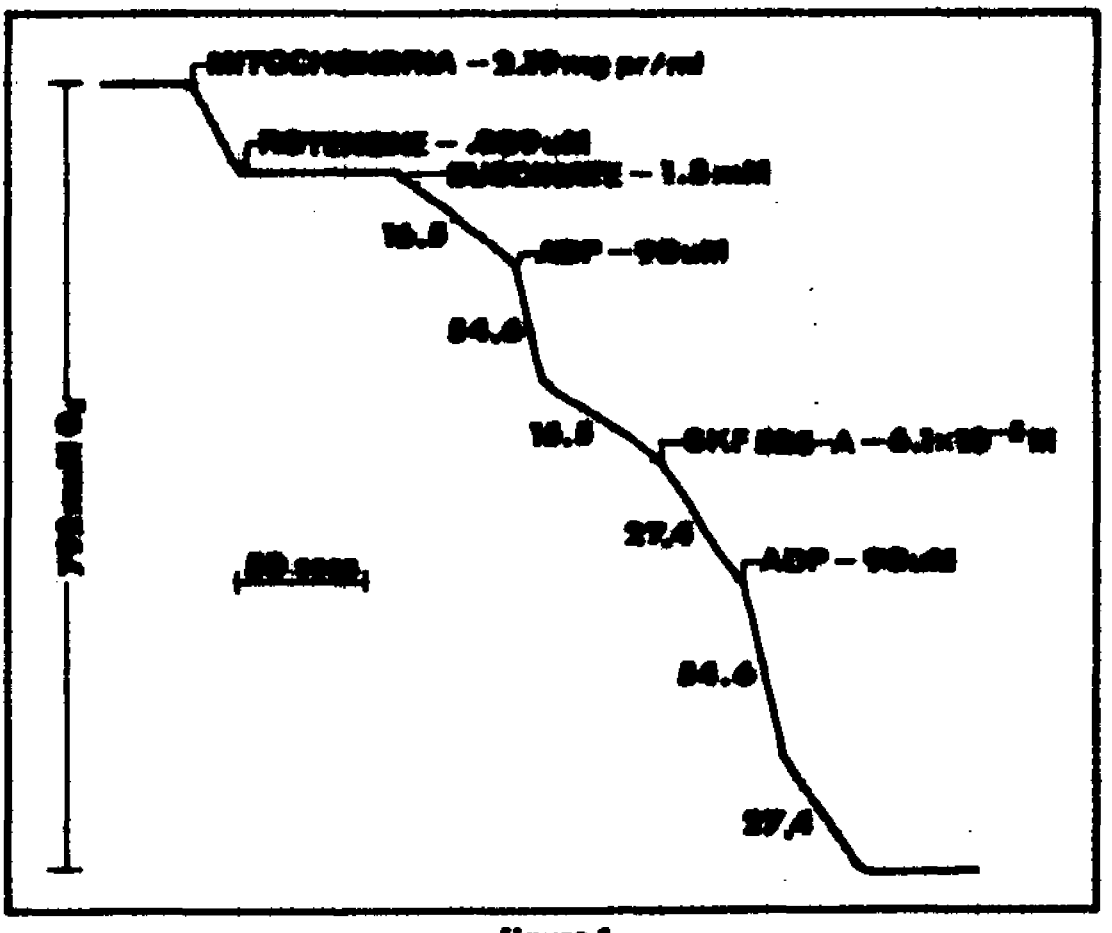


figure 1

FIGURE 2

Graphical representation of data of the type in table 2. Experimental conditions as in figure 1.

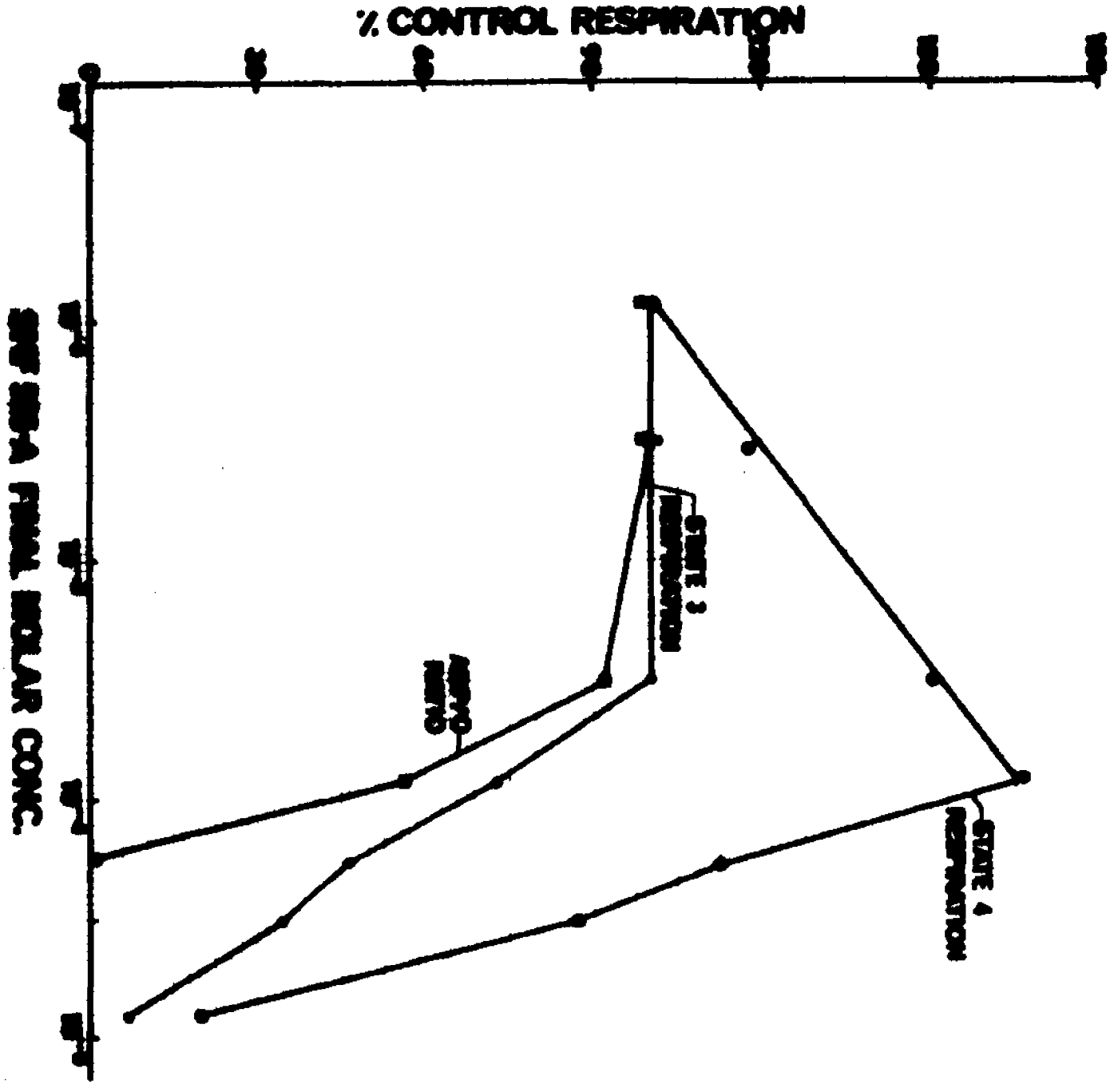

% CONTROL RESPIRATION

FIGURE 3

The interaction of SKF525-A with oligomycin during respiration of rat liver mitochondria under conditions identical with those in Figure 1.

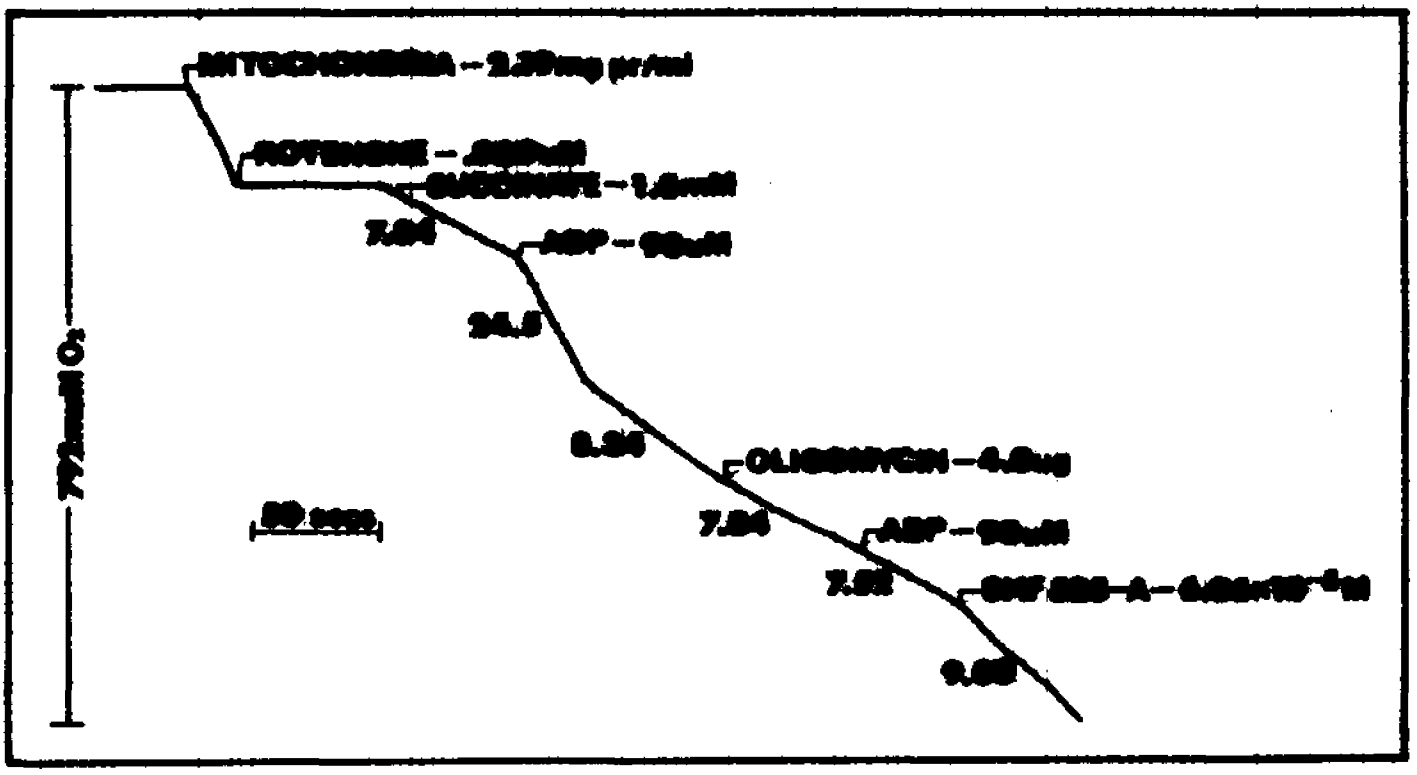


figure 3

FIGURE 4

Respiration of sonically produced submitochondrial particles under conditions described above. 0.72 mg/ml submitochondrial protein present.

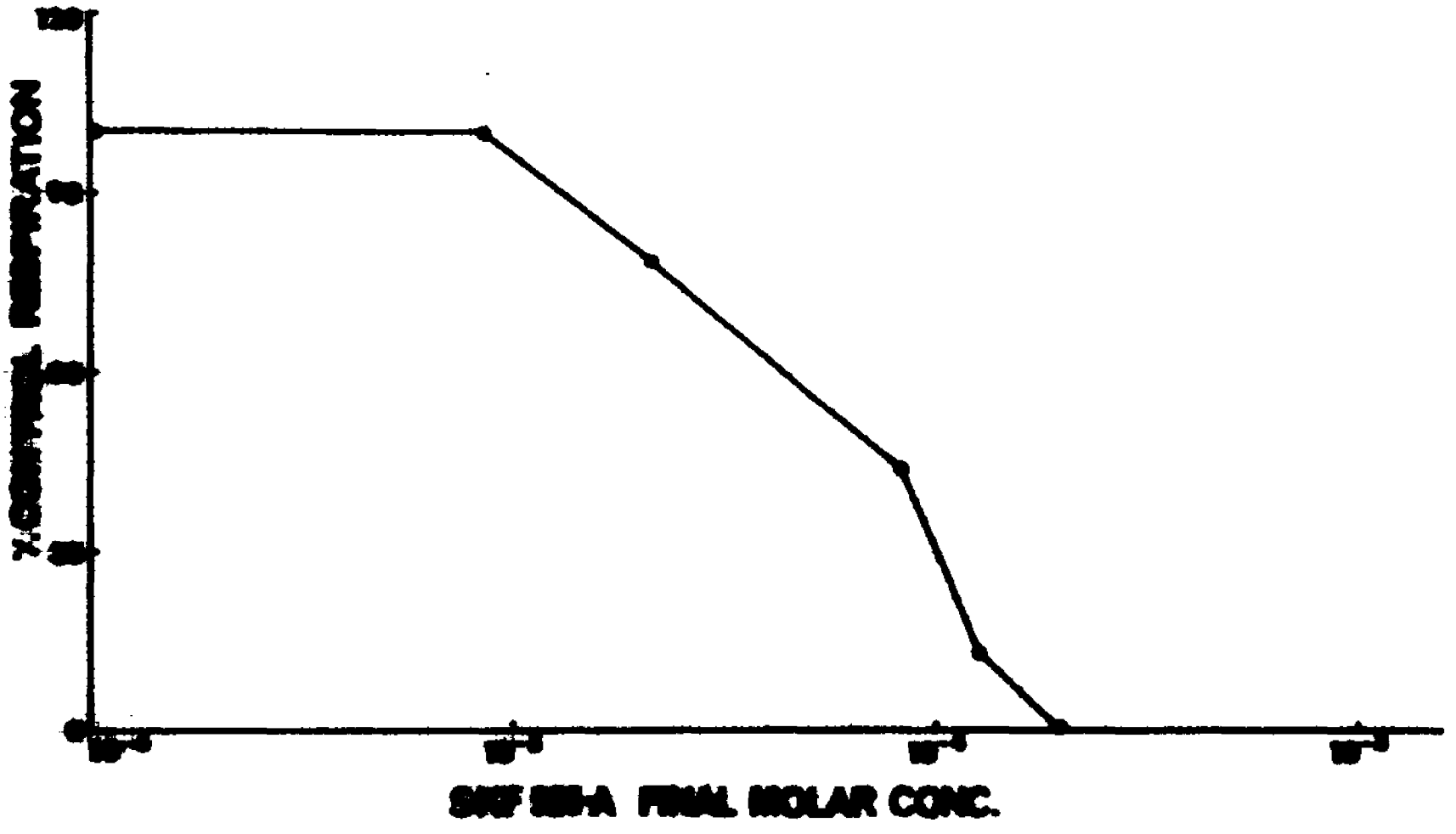


FIGURE 5

Relationship between mitochondrial concentration and the respiratory rate. Experimental conditions as in Figure 1. The substrate was 1.8mM succinate and the SKF525-A concentration was 30 uM.

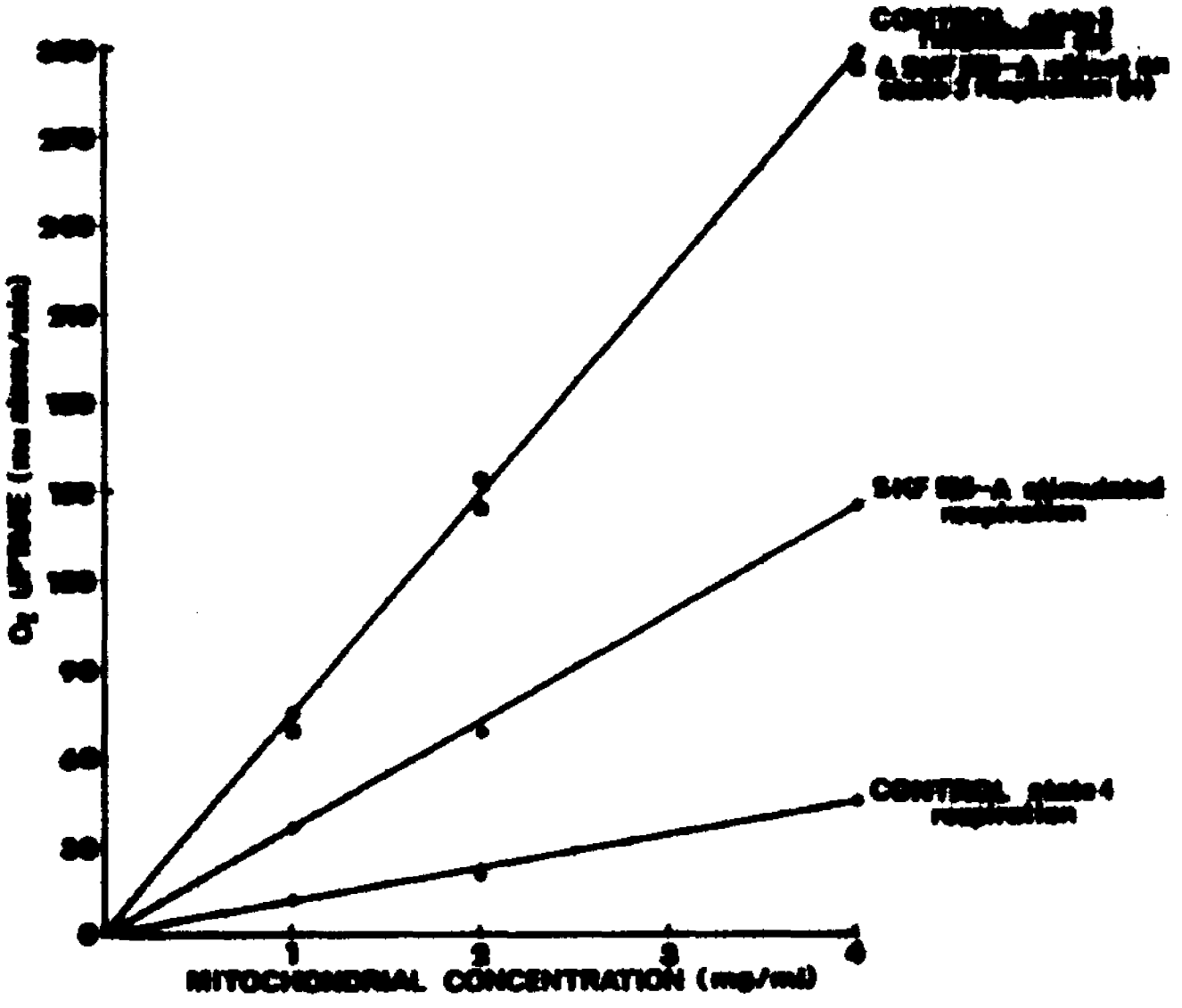

1
2
3
4
MITOCHONDRIAL CONCENTRATION (mg/ml)

FIGURE 6

Inhibition of mitochondrial respiration induced by higher concentrations of SKF525-A and the resumption of respiration using ascorbate and TMPD. Experimental conditions as in Figure 1. Oxygen uptake expressed as (mumoles O_2/min)/mg mitochondrial protein and shown to the left of the slopes on the record.

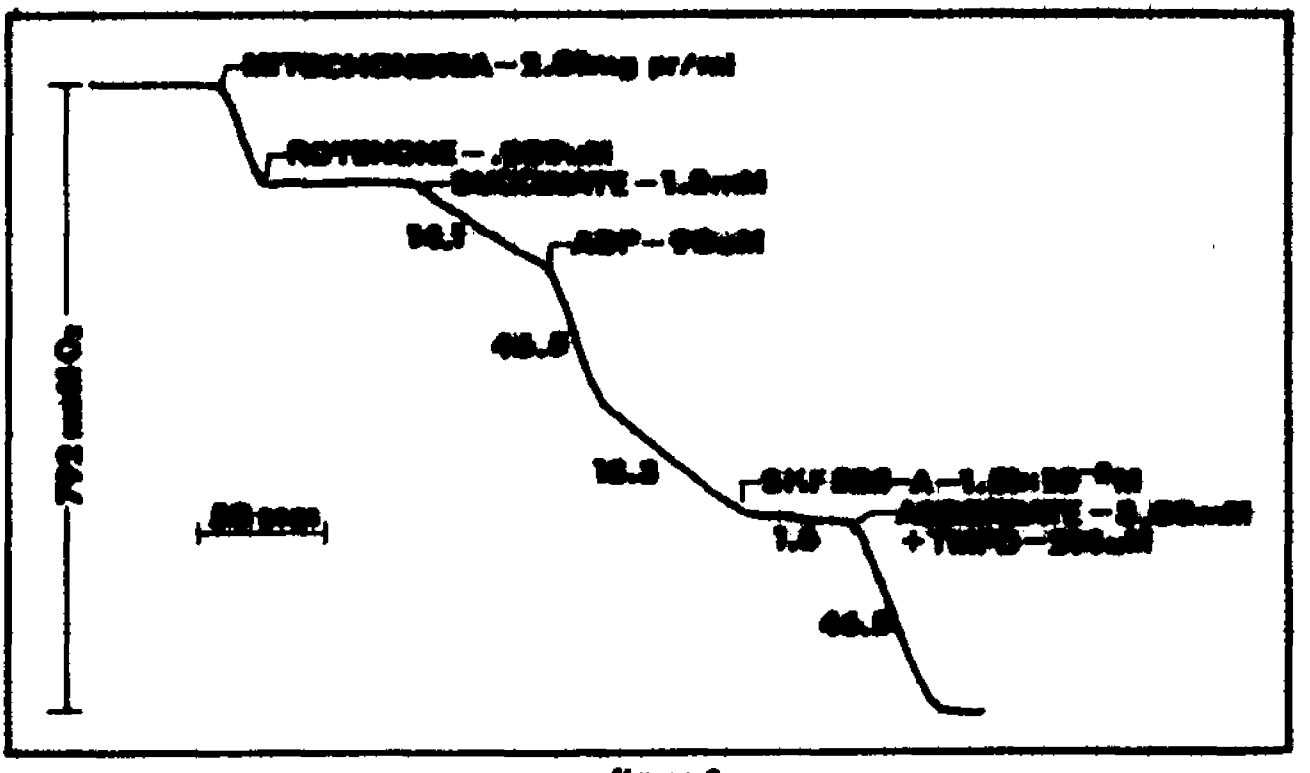


figure 6

FIGURE 7

The interaction of Ca^{++} and ADP with rat liver mitochondria respiring with and without oligomycin. Experimental conditions described in Methods.

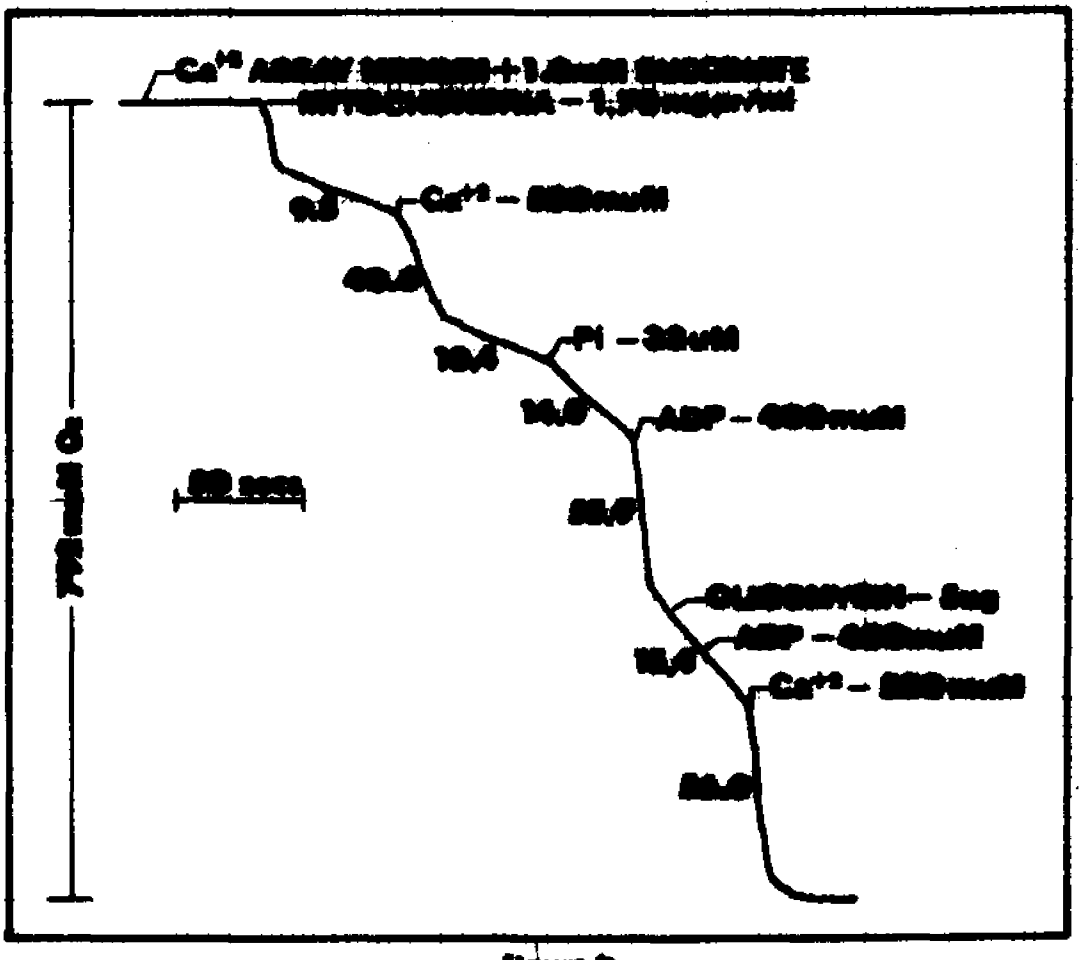


Figure 9

PLATE 1

Mitochondria isolated in sucrose-HEPES isolation media and respiring in state 4 in the succinate medium described in Methods. Magnification = 15,960X.

PLATE 2

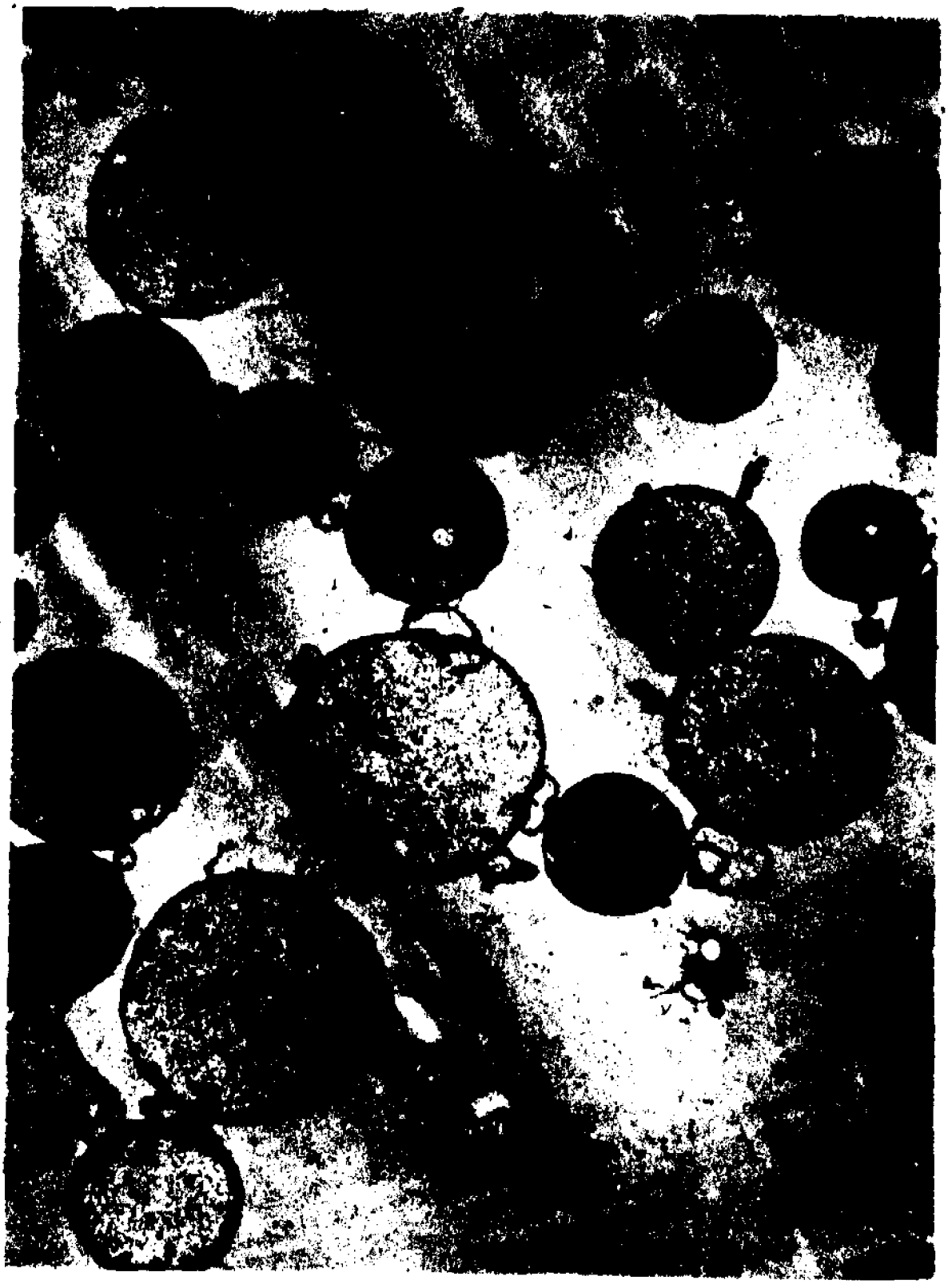
Mitochondria respiring as in plate 1 except 20 seconds after the addition of 9.09×10^{-5}M SKF525-A. Magnification = 22,344.

PLATE 3

Mitochondria respiring as in plate 1 except 1 minute after the addition of $9,09 \times 10^{-5}$M SKF525-A. Magnification = 19,565.

PLATE 4

Mitochondria respiring as in plate 1 except 3 minutes after the addition of 9.09×10^{-5}M SKF525-A. Magnification = 23,940.



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