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EFFECTS OF SC-26096 [2-METHYL-3-OXO-2-AZABICYCLO (2.2.2.) OCTAN-6-*EXO*-YL 5-(4-BIPHENYLYL)-3-METHYL VALERATE], A NEW HYPOLIPIDEMIC AGENT, ON MITOCHONDRIAL RESPIRATION AND OXIDATIVE PHOSPHORYLATION

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Abstract—The effects of SC-26096 [2-methyl-3-oxo-2-azabicyclo (2.2.2.) octan-6-*exo*-yl 5-(4-biphenylyl)-3-methyl valerate] on respiration and oxidative phosphorylation of rat liver mitochondria were studied *in vitro*. It was found that SC-26096 is an inhibitor of electron transport. In preparations of intact and sonicated mitochondria, the drug effectively inhibited NAD-linked respiration at low concentration and inhibited succinate-linked respiration at much higher concentration. In intact mitochondria, dinitrophenol partially reversed the inhibition of NAD-linked state 3 repiration but did not reverse the inhibition of succinate-linked respiration. At the SC-26096 concentrations which were required to inhibit succinate oxidation, ADP:O ratio of phosphorylation site III was lowered and state 4 oxidation rate and ATPase activities were raised. At the drug concentrations which were tested, SC-26096 did not alter the ADP:O ratio of phosphorylation site II. 3-Hydroxybutyrate oxidation by mitochondria which were pretreated with SC-26096 remained inhibited after washing with 0·25 M sucrose, but this inhibition was relieved by adding albumin. The effects of SC-26096 were primarily dependent upon the ratio of drug:mitochondrial protein and not upon the initial molarity of the drug in the incubation medium. The results suggest that SC-26096 is bound to mitochondria *in vitro* and that the bound drug produces the mitochondrial effects. At least two sites of inhibition, within the respiratory chain, are indicated. One site lies between the interaction of NADH with NADH dehydrogenase and the point at which electrons from succinate oxidation enter the electron transport system. A second, less sensitive, site lies between the interaction of succinate with succinate dehydrogenase and cytochrome c. A third inhibitory site appears to be present in the site I phosphorylation system at or distal to the dinitrophenol-sensitive site.

THE HYPOLIPIDEMIC agents, clofibrate (*p*-ethyl-chlorophenoxyisobutyrate),[1–3] DPTH (5,5-diphenyl-2-thiohydantoin)[4] and AY-9944 [*trans*-1, 4-bis-(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride],[1] have previously been shown to be inhibitors of mitochondrial electron transport and uncouplers of oxidative phosphorylation. At present, the importance of these properties, relative to both toxicity and therapeutic hypolipidemic action, is unknown. However, it is possible that the mitochondrial effects, which were observed *in vitro*, might also occur *in vivo*. In this regard, clofibrate administration has been shown to alter mitochondrial ultrastructure[5] and decrease the respiratory quotient and oxygen consumption of rats.[6]

Recently, our laboratories discovered a new hypolipidemic agent, SC-26096 [2-methyl-3-oxo-2-azabicyclo (2.2.2) octan-6-*exo*-yl 5-(4-biphenyl)-3-methyl valerate] (J. E. Miller, R. E. Ranney, E. Muir and J. H. Dygos, unpublished observations). In microbiological experiments, this agent was found to be a metabolic inhibitor which markedly increased the synthesis of lactic acid by cultures of both L cells and chicken embryo cells (W. R. Smith and C. R. Mackerer, unpublished observations). It was, therefore, of interest to determine if SC-26096 is an inhibitor of oxidative phosphorylation.

The results of the experiments reported herein show that SC-26096, at low concentrations, is an inhibitor of mitochondrial respiration and, at much higher concentrations, an uncoupler of oxidative phosphorylation. NADH-linked oxidation was inhibited at considerably lower levels of SC-26096 than have been reported to be required of clofibrate,[3] DPTH[4] and AY-9944.[1]

METHODS

Animals. Male Charles River CD rats (body wt 250-300 g) were used for all experiments. These rats were fed a commercial pelleted diet [Rockland Mouse/Rat Diet (Complete)], *ad lib.*

Isolation of intact rat liver mitochondria. Mitochondria were freshly isolated, by a previously described method,[7] and suspended in 0·25 M sucrose at a concentration of 25–27 mg protein/ml. Mitochondrial protein was determined by a biuret procedure.[8]

Preparation of mitochondrial fragments. Five ml of mitochondrial suspension was sonified for 1 min at 50 watts. The sonifier (Branson Sonic Power Co.) was equipped with a 0·5-in. disruptor horn.

Oxygen consumption. Oxygen consumption of intact mitochondria was determined polarographically at 30° as previously described.[3] The incubation media (1·92 ml, pH 7·4) contained the following constituents at the indicated concentrations: Tris, 65 mM; KCl, 75 mM; $MgCl_2$, 5 mM; phosphate, 12 mM; EDTA, 1 mM; and substrate, 8·6 or 17·2 mM. The substrates were: DL-3-hydroxybutyrate, 17·2 mM; succinate, 8·6 mM; and ascorbate, 8·6 mM. For the substrates and EDTA, potassium was the counterion. Tris and phosphate buffers (pH 7·4) were prepared by mixing aliquots of stock solutions containing Tris and Tris–HCl, and KH_2PO_4 and K_2HPO_4, respectively. When ascorbate was used, 0·2 μmole of tetramethylphenylenediamine (TMPD)/mg of mitochondrial protein was included in the medium to facilitate transfer of electrons from ascorbate to the respiratory chain.[9] State 3 respiration was initiated by adding ADP. DNP (2,4-dinitrophenol) at $1·6 \times 10^{-5}$ M was used to uncouple oxidative phosphorylation. When sonicated mitochondria were used, respiration was uncontrolled and, therefore, ADP and DNP were not added. ADP:O ratio and the rates of oxygen consumption in states 3 and 4 were determined by the methods of Chance and Williams.[10,11]

ATPase activity. Rat liver mitochondrial ATPase activity was determined by the method of Katyal *et al.*[1]

SC-26096 solution. SC-26096 is rather insoluble in H_2O and buffer solutions and, therefore, had to be dissolved in an organic solvent. Propylene glycol was chosen

because a 0·1 M stock solution of SC-26096 could be prepared and additions of propylene glycol alone, at concentrations of up to 1% (v/v) of the various reaction mixtures, did not affect any of the experimental parameters.

Chemicals. Dr. J. H. Dygos of Searle Laboratories synthesized the SC-26096; lot II-136 was used for all studies. SC-26096 (mol. wt 406) is an odorless, white, crystalline powder which has a melting point of 74–75·5° and an absorbance maximum (in methanol) at 252 nm. Lot II-136 was approximately 99·5 per cent pure; three unidentified organic impurities, totaling less than 0·5 per cent were present. Heavy metal content was less than 20 ppm. Organic reagents were from Sigma Chemical Co., St. Louis, Mo., and inorganic salts from Mallinckrodt Chemical Co., St. Louis. Fatty acid free, bovine serum albumin was prepared from Pentex, Fraction V Powder (Miles Laboratories, Inc., Kankakee, Ill.).[12,13]

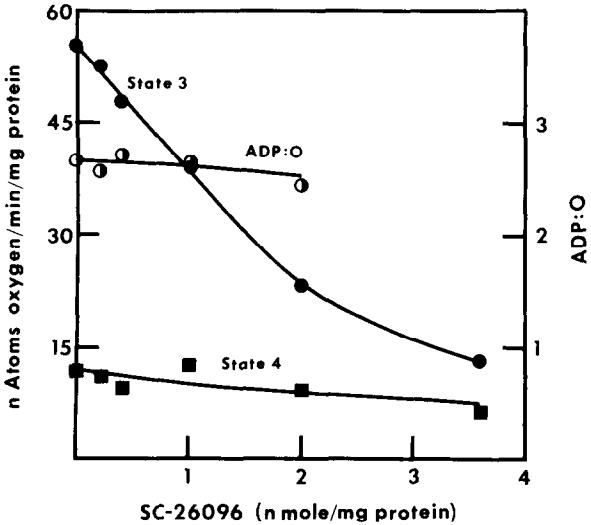

FIG. 1. Effects of SC-26096 on oxygen consumption and ADP:O ratio of intact mitochondria with 3-hydroxybutyrate as substrate. Reactions were initiated by adding 5·0 mg mitochondrial protein and 610 nmoles ADP. Each value is the mean of nine replicates.

RESULTS

Effect of SC-26096 on oxidation of 3-hydroxybutyrate, succinate and ascorbate (+ TMPD) by intact mitochondria. Figure 1 shows the effects of SC-26096 on 3-hydroxybutyrate oxidation in states 3 and 4, and on ADP:O ratio. State 3 oxygen consumption was inhibited by SC-26096, in a dose-dependent manner, with 30 per cent inhibition occurring at a concentration of approximately 1·2 nmoles/mg of protein. State 4 oxidation was also inhibited but only at levels above 2·0 nmoles/mg of protein. ADP:O ratio was not altered by concentrations of up to 2·0 nmoles/mg of protein.

Addition of DNP to inhibited mitochondria produced a partial reversal of inhibition and increased the rate of respiration (Fig. 2). In the absence of inhibitor, the state 3 rate was faster than the uncoupled rate, but the converse was true in the presence of inhibitor.

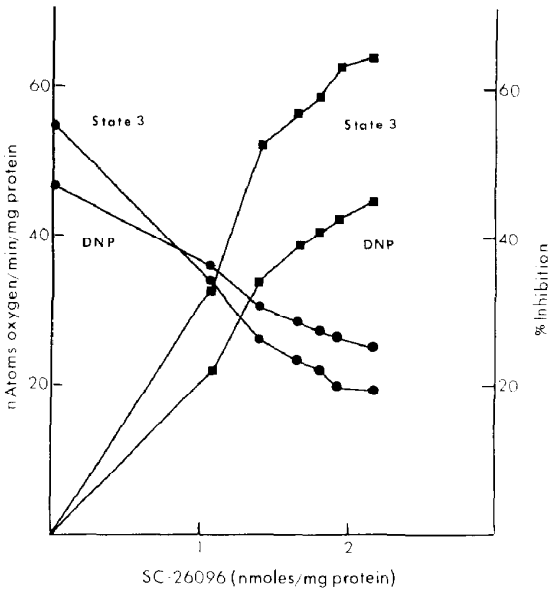

FIG. 2. Effects of SC-26096 on state 3 oxygen consumption of intact mitochondria in the presence and absence of DNP. The order of addition of components was: incubation medium, mitochondria (5·0 mg), drug in vehicle, ADP (610 nmoles) and DNP ($1·6 \times 10^{-5}$M). Each value is the mean of three replicates.

The effects of SC-26096 on succinate oxidation in states 3 and 4, and on ADP:O ratios are shown in Fig. 3. As in the 3-hydroxybutyrate system, SC-26096 inhibited succinate-linked state 3 respiration in a dose-dependent manner, but the required concentrations were considerably higher; 30 per cent inhibition occurred at approximately 60 nmoles/mg of protein. Progressive increases in SC-26096 concentration,

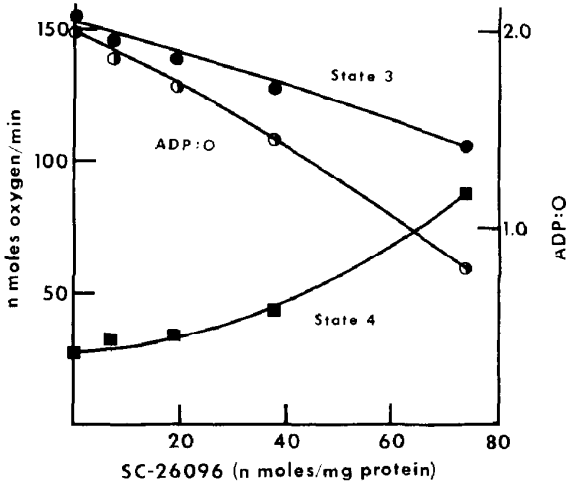

FIG. 3. Effects of SC-26096 on oxygen consumption and ADP:O ratio of intact mitochondria with succinate as substrate. Reactions were initiated by adding 2·7 mg mitochondrial protein and 600 nmoles ADP. Each value is the mean of three replicates.

over the range of 20–74 nmoles/mg of protein, led to elevated state 4 respiration rates and lowered ADP:O ratios.

The partial release of SC-26096 inhibition by DNP, which was observed in the 3-hydroxybutyrate system, was not seen when succinate was used as the substrate. Rather, SC-26096 inhibited state 3 and DNP-stimulated respirations to the same degree.

The effects of SC-26096 on ascorbate (+TMPD) oxidation in states 3 and 4, and on ADP:O ratio are shown in Fig. 4. SC-26096 was a very weak inhibitor of this state 3 respiration, and at 80 nmoles/mg of protein there was only a 13 per cent reduction in oxygen consumption. As in the succinate system, SC-26096 in the range of 20–74 nmoles/mg of protein elevated the rate of state 4 respiration but lowered the ADP:O ratio. SC-26096 inhibited state 3 and DNP-stimulated respirations to the same degree.

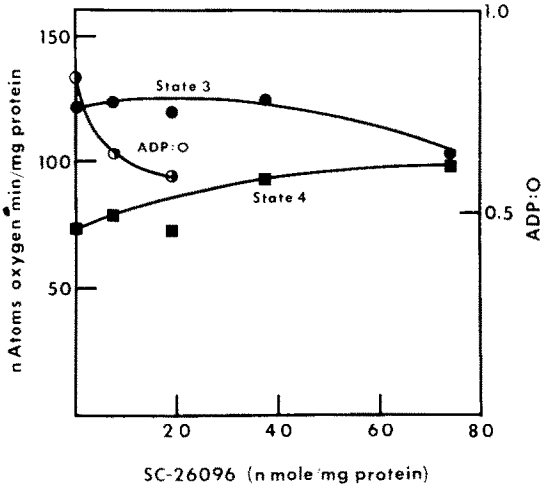

FIG. 4. Effects of SC-26096 on oxygen consumption and ADP:O ratio of intact mitochondria with ascorbate (+TMPD) as substrate. Reactions were initiated by adding 2·7 mg protein and 565 nmoles ADP. Each value is the mean of three replicates.

Effect of SC-26096 on oxidation of NADH, succinate and ascorbate (+TMPD) by sonicated mitochondria. In order to determine if SC-26096 inhibited state 3 respiration via direct action on the respiratory chain, experiments were performed using sonicated mitochondria. Sonication disrupts mitochondrial membranes and exposes the inner membrane surfaces to the external medium; this procedure reduces the probability that permeability barriers, between medium and matrix, will affect the respiratory rates.

With NADH, succinate and ascorbate (+TMPD) as substrates, respiration appeared to be entirely uncoupled from oxidative phosphorylation and, therefore, additions of ADP and DNP failed to increase the rate of oxygen consumption. NADH was used in place of 3-hydroxybutyrate in these studies because respiration, linked to 3-hydroxybutyrate oxidation, was very slow in the absence of added NAD or NADH. Presumably, the sonication procedure caused the soluble, but compartmented, cofactors to be freely dissolved and diluted in the incubation medium. Eli-

TABLE 1. INHIBITORY EFFECTS OF SC-26096 ON RESPIRATION OF SONICATED RAT LIVER MITOCHONDRIA*

Substrate	SC-26096 (nmoles/mg protein)	Inhibition of oxygen consumption (%)
NADH	0·19	37·5
	0·37	30·2
	0·74	52·2
	1·90	71·7
	3·70	85·6
	7·40	92·4
Succinate	37·00	13·0
	93·00	34·9
	185·00	79·5
	370·00	96·7
Ascorbate (+TMPD)	18·50	11·4
	185·00	0·0
	1850·00	0·0

* Oxygen consumption was determined polarographically as described under Methods. Substrate concentration was 11 mM. Each value is the mean of three replicate experiments.

mination of the 3-hydroxybutyrate dehydrogenase step did not seem important, since SC-26096, at concentrations of up to 4 nmoles/mg of protein, did not inhibit the enzyme itself.

The results of our experiments with sonicated mitochondria are summarized in Table 1. It can be seen that SC-26096 inhibited succinate- and NADH-linked oxidations by sonicated as well as by intact mitochondria. However, ascorbate (+TMPD) oxidation was not inhibited by SC-26096 at concentrations up to 1·85 μmoles/mg of protein.

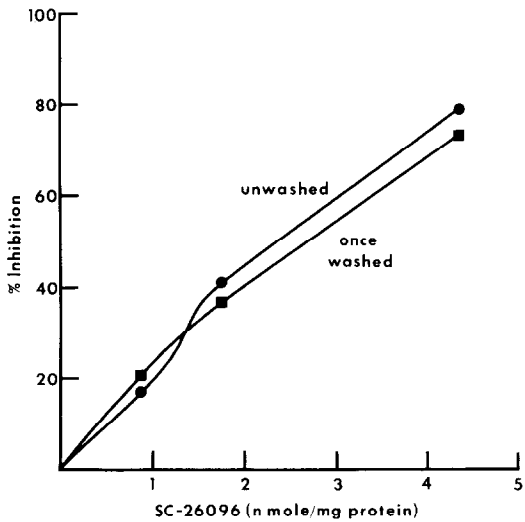

FIG. 5. Effects of SC-26096 pretreatment and subsequent washing on state 3 oxygen consumption with 3-hydroxybutyrate as substrate. The washing procedure is described in the text. Each value is the mean of three replicates.

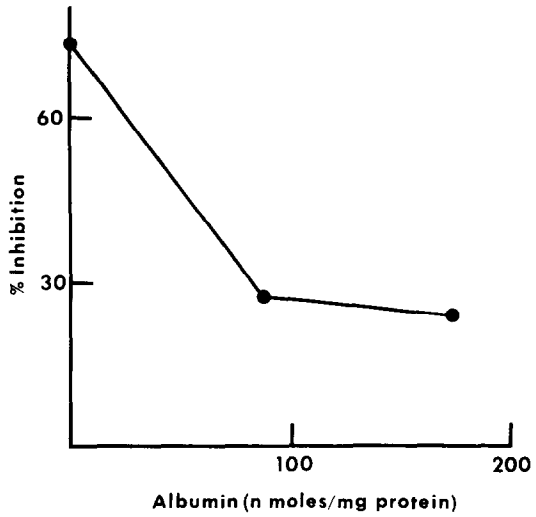

FIG. 6. Effects of fatty acid free, bovine serum albumin on SC-26096 inhibition of state 3 oxidation with 3-hydroxybutyrate as substrate. The experimental procedure is described in the text. Each value is the mean of three replicates.

Binding of SC-26096 to mitochondria. SC-26096, at three different levels, was added to 5-ml aliquots of mitochondria. These were incubated at 0° for 10 min and then centrifuged at 15,000 *g* for 5 min. The mitochondrial pellet was washed once with 5 ml of 0·25 M sucrose and resuspended. State 3 oxidations of 3-hydroxybutyrate, by the washed and unwashed mitochondria, were compared. The plots of mitochondrial inhibition, as a function of SC-26096 concentration (Fig. 5), were similar, indicating that the drug was bound to the mitochondria and not removed by washing with a sucrose medium.

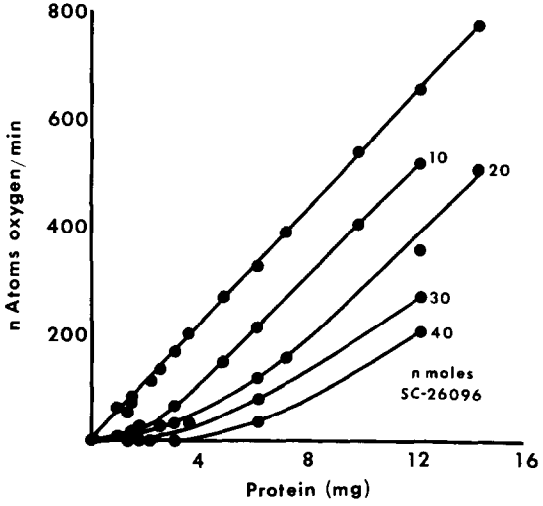

FIG. 7. Effects of increasing mitochondrial protein concentrations on the DNP-stimulated oxidation rates of intact mitochondria at several concentrations of SC-26096. 3-Hydroxybutyrate was used as substrate. Each value is the mean of two replicates.

Reversal of SC-26096 inhibition by albumin. Albumin has been shown to reverse the inhibitory effects of nordihydroguaiaretic acid[14] and the uncoupling effects of dicumarol[15] on oxidative phosphorylation. It was, therefore, of interest to determine if the mitochondrial effects of SC-26096 could also be reversed by albumin. Mitochondria were preincubated with SC-26096 (4·4 nmoles/mg of protein) and washed once, as described above. Mitochondria were added to the incubation medium, in the oxygraph chamber, followed by ADP, and sufficient time was allowed for an accurate measurement of oxygen consumption in state 3. Then, two successive additions of albumin were made, and their separate effects on state 3 were determined. The results of this experiment are shown in Fig. 6. It can be seen that albumin reversed, to a substantial degree, the inhibitory effects of SC-26096. Other experiments revealed that it did not matter whether the albumin was added prior to or after SC-26096.

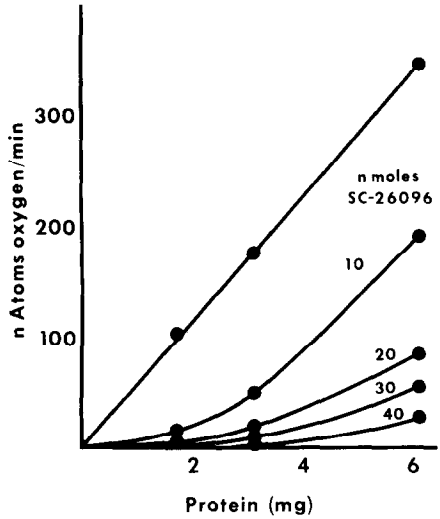

FIG. 8. Effects of increasing mitochondrial protein concentration on the state 3 oxidation rates of intact mitochondria at several concentrations of SC-26096. 3-Hydroxybutyrate was used as substrate. Each value is the mean of two replicates.

Inhibitory effects of SC-26096 as a function of mitochondrial protein concentration. Since SC-26096 appeared to be bound to mitochondria, it was expected that effects would be more closely related to the ratio of drug : mitochondrial protein than to the initial molarity of drug in the incubation medium. This was found to be the case; as shown in Figs. 7–9, inhibitions of ADP and DNP-stimulated respiration, with 3-hydroxybutyrate as substrate, were dependent upon both the amount of drug and the amount of mitochondrial protein present in the system. Progressive elevation of the mitochondrial protein level, at constant drug concentration, initially caused increasing amounts of inhibition; however, after a critical drug : protein ratio was reached, further mitochondrial additions did not alter the degree of inhibition. These effects were seen even at SC-26096 concentrations which inhibited respiration only slightly (Fig. 9). The degree of inhibition was the same regardless of the sequence in which mitochondria and SC-26096 were added.

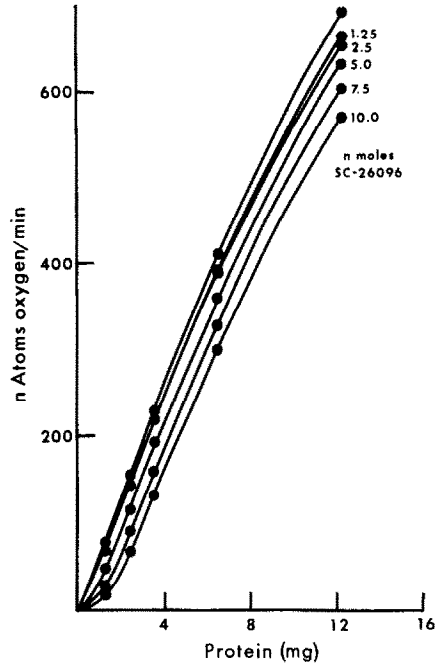

FIG. 9. Effects of increasing mitochondrial protein concentration on the DNP- stimulated oxidation rates of intact mitochondria at low concentrations of SC-26096. 3-Hydroxybutyrate was used as substrate. Each value is the mean of three replicates.

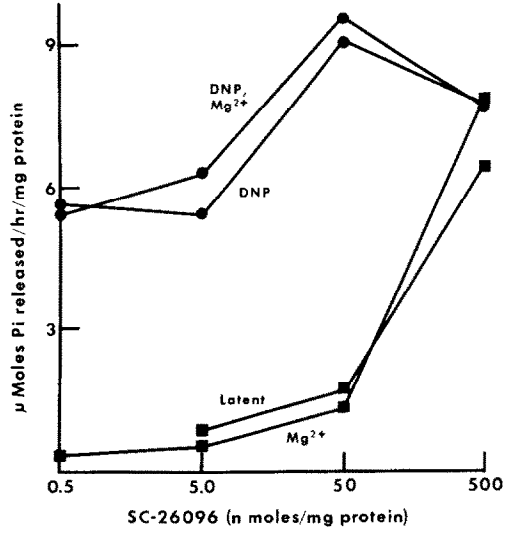

FIG. 10. Effects of SC-26096 on mitochondrial ATPases. The basic incubation medium (pH 7·4) contained 6 mM ATP, 10 mM Tris–HCl, 75 mM KCl and 4 mg protein. Further additions to the medium are as indicated. Total volume was 2 ml. Incubation was for 15 min at 30°. Each value is the mean of three replicates.

Effects of SC-26096 on ATPase activity. Uncouplers of oxidative phosphorylation usually cause an increase in mitochondrial ATPase activity, presumably via reversal of the terminal phosphorylating steps.[16] DNP, the uncoupler used for our present studies, is a classical example of an uncoupler which produces this effect.[17,18] Since SC-26096 lowered ADP:O ratios (Figs. 3 and 4), it was likely that ATPase would also be stimulated. The effect of increasing concentrations of SC-26096 on ATPase activity is presented in Fig. 10. SC-26096 markedly increased the activity of endogenous (latent) ATPase and further increased the activity of DNP-stimulated ATPase. Addition of Mg^{2+} did not stimulate ATPase activity, either in the presence or absence of DNP, and did not alter the effect of SC-26096. The concentration range of SC-26096 (5–500 nmoles/mg of protein) over which succinate and ascorbate (+ TMPD)-linked state 4 respirations were stimulated, and ADP:O ratios were decreased, corresponded to the range over which ATPase activity was increased.

DISCUSSION

Mitochondrial inhibition. Our results show that SC-26096 is an inhibitor of mitochondrial respiration. The differential effects of the drug, with respect to concentrations required for inhibiting the various substrate-linked oxidations of both intact and sonicated liver mitochondria (Figs. 1, 3 and 4 and Table 1), suggest that there are at least two specific sites of inhibition within the respiratory chain. These sites are shown in Fig. 11, which presents a schematic diagram of the respiratory chain linked to the energy transfer system. At least one site exists between the interaction of NADH with NADH dehydrogenase and the point at which electrons from succinate oxidation enter the electron transport chain. This must be the case since inhibition of NAD-linked oxidations occurred at drug concentrations which did not affect succinate oxidation (Figs. 1 and 3 and Table 1). At least one other, less sensitive, site exists between the interaction of succinate with succinate dehydrogenase and cytochrome c, the probable point at which electrons from ascorbate (+TMPD) oxidation enter the electron transport chain.[21] Lack of inhibition with ascorbate (+

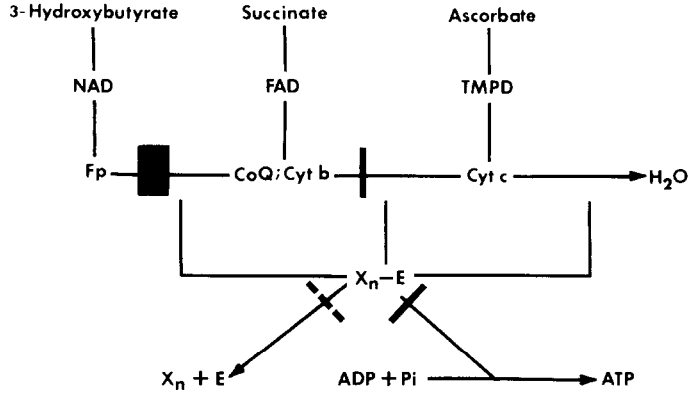

FIG. 11. Hypothetical scheme of the energy transfer system, linked to the electron transport chain, and the sites of SC-26096 action. Abbreviations: Fp, NADH dehydrogenase; CoQ, coenzyme Q; Cyt, cytochrome; TMPD, tetramethylphenylene diamine; X_n, hypothetic energy transfer carriers; E, energy. The sites of SC-26096 are at or before the sites indicated by the bars. The widths of the bars represent relative activity (see Lee and Ernster[19] and Hoch[20]); a solid bar represents inhibition and a dashed bar stimulation.

TMPD) as substrate (Fig. 4 and Table 1) demonstrated the absence of an inhibitory site distal to cytochrome c.

The inhibitory effects of SC-26096 on state 3 oxygen consumption of the 3-hydroxybutyrate system were partially reversed by addition of DNP. This indicates that SC-26096 is also an inhibitor of the energy transfer system, of the first phosphorylation site, at a point at or distal to the DNP-sensitive site ($Xn - E \rightarrow Xn + E$; Fig. 11). Inhibition exerted distal to the DNP-sensitive site is reversed by addition of DNP.[14,22,23]

Since SC-26096 inhibited NAD-linked oxidations and succinate oxidations by both intact (Figs. 1 and 3) and sonicated mitochondria (Table 1), it can be assumed that the major inhibitory effects did not occur by altering membrane permeabilities. In fact, SC-26096 was active at lower concentrations with sonicated than with intact mitochondria.

Mitochondrial oxidative phosphorylation. Uncoupling of oxidation from phosphorylation causes energy to be released as heat rather than conserved as ATP.[24] This decreased efficiency results in more oxygen consumption per quantity of ADP phosphorylated. In our systems *in vitro* uncoupling is indicated by a fall in the ADP:O ratio which is usually accompanied by increased state 4 respiration rate and increased ATPase activity. SC-26096 produced all of these effects in the concentration range (5–74 nmoles/mg of protein) required to inhibit succinate-linked respiration (Figs. 3 and 10) and, therefore, can be assumed to have uncoupling activity. However, SC-26096 did not uncouple oxidative phosphorylation at the lower concentrations which markedly inhibited 3-hydroxybutyrate oxidation (Fig. 1).

Uncoupling did not occur equally at phosphorylation sites II and III. The ADP:O ratio of each individual phosphorylation site has been calculated via direct substraction,[25] and we have used this procedure to estimate the ADP:O ratios associated with phosphorylation sites II and III. As indicated by the results of Table 2, phosphorylation associated with site III is more sensitive to uncoupling than is the phosphorylation associated with site II. DNP also produces a greater uncoupling of site III than of site II.[25] ADP:O ratio of site I could not be calculated by this method because at the required concentrations of SC-26096, 3-hydroxybutyrate oxidation was almost completely inhibited.

Mitochondrial binding of SC-26096. The lack of effect of washing on SC-26096-inhibited mitochondria (Fig. 5) and the dependence of the degree of inhibition on the amount of mitochondria present (Figs. 7–9) indicate that the drug is bound to mito-

TABLE 2. EFFECT OF SC-26096 ON COUPLING AT PHOSPHORYLATION SITES II AND III*

SC-26096 added (nmoles/mg protein)	Site II ADP:O	Site II Inhibition (%)	Site III ADP:O	Site III Inhibition (%)
0·0	1·03		0·833	
7·4	1·09	0	0·642	23
18·5	1·02	0	0·598	28

* ADP:O ratio of site II was calculated by substracting the ADP:O ratios of the ascorbate (+TMPD) system (Fig. 4) from those of the succinate system (Fig. 3). The ADP:O ratios of the ascorbate (+TMPD) system were assumed to represent those of site III. Each value is the mean of three replicate experiments.

chondria. SC-26096 is extremely insoluble in H_2O but is soluble in long chain alcohols and in oils, and these facts raise the possibility that binding may be related to solubility in mitochondrial lipids. If SC-26096 is very soluble in these lipids, the washing of pretreated mitochondria with aqueous solutions would not be expected to remove appreciable amounts of drug. However, the inhibitory effects on respiration are probably produced via binding to particular sites within the respiratory chain and are not dependent upon the concentration of free drug in the lipid phase. If this is not the case, increasing the amount of mitochondria, at constant drug concentration, would be expected to reverse the inhibitory effects because the increased amount of lipid would produce a dilution. When this experiment was performed, no reversal of inhibition was observed (Figs. 7–9), even at very low concentrations of SC-26096 which inhibited 3-hydroxybutyrate oxidation only slightly (Fig. 9).

TABLE 3. EFFECTS OF SC-26096, CLOFIBRATE, DPTH and AY-9944 ON INHIBITION OF NAD-LINKED RESPIRATION OF LIVER MITOCHONDRIA

Source of data	Drug			Inhibition of state 3 (%)
	Name	Concentration (nmoles/mg protein)	Substrate	
Present report	SC-26096	1·3	3-Hydroxybutyrate	40
Mackerer *et al.*[3]	Clofibrate	90	3-Hydroxybutyrate	40
Mehlman *et al.*[4]	DPTH	170*	Pyruvate plus malate	41
Katyal *et al.*[1]	AY-9944	72*	Glutamate	42

* These concentrations were calculated utilizing the authors' values for the amounts of drug and protein added and from the volumes of the reaction mixtures. AY-9944 was assumed to have a mol. wt of 464.

Inhibition of NAD-linked respiration by hypolipidemic agents. As shown in Table 3, SC-26096 inhibited NAD-linked respiration at concentrations considerably below those reported to be required of clofibrate,[3] DPTH[4] and AY-9944.[1] At present, it is not known whether the mitochondrial effects of the various hypolipidemic agents are of any significance, *in vivo*.

REFERENCES

1. S. L. KATYAL, J. SAHA and J. J. KABARA. *Biochem. Pharmac.* **21**, 747 (1972).
2. S. R. PANINI. *Biochem. J.* **128**, 50P (1972).
3. G. R. MACKERER, J. R. HAETTINGER and T. C. HUTSELL. *Biochem. Pharmac.* **22**, 513 (1973).
4. M. A. MEHLMAN, R. B. TOBIN, W. R. RUEGAMER and M. W. MADAPPALLY. *Biochem. Pharmac.* **21**, 2031 (1972).
5. G. E. PAGET, *J. Atheroscler. Res.* **3**, 729 (1963).
6. J. M. THORP. *J. Atheroscler. Res.* **3**, 737 (1963).
7. D. JOHNSON and H. A. LARDY, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), Vol. 10, p. 94. Academic Press, New York (1967).
8. A. G. GORNALL, C. S. BARDAWILL and M. M. DAVID, *J. biol. Chem.* **177**, 751 (1949).
9. E. E. JACOBS, *Biochem. biophys. Res. Commun.* **3**, 536 (1960).
10. B. CHANCE and G. R. WILLIAMS, *J. biol. Chem.* **217**, 383 (1955).
11. B. CHANCE and G. R. WILLIAMS, *Adv. Enzymol.* **17**, 88 (1956).
12. R. F. CHEN, *J. biol. Chem.* **242**, 173 (1967).
13. R. W. HANSON and F. J. BALLARD, *J. Lipid Res.* **9**, 667 (1968).
14. C. BHUVANESWARAN and K. DAKSHINAMURTI, *Biochemistry* **11**, 85 (1972).
15. E. C. WEINBACH and J. GARBUS, *J. biol. Chem.* **241**, 3708 (1966).
16. H. C. HEMKER, *Biochim. biophys. Acta* **81**, 1 (1964).
17. H. A. LARDY and H. WELLMAN, *J. biol. Chem.* **195**, 215 (1952).
18. H. A. LARDY and H. WELLMAN, *J. biol. Chem.* **201**, 357 (1953).

19. C. P. LEE and L. ERNSTER, in *Regulation of Metabolic Processes in Mitochondria* (Eds. J. M. TAGER, S. PAPA, E. QUAGLIARIELLO and E. C. SLATER), p. 218. Elsevier, New York (1966).
20. F. L. HOCH, *Energy Transformations in Mammals: Regulatory Mechanisms*, p. 43. W. B. Saunders, Philadelphia (1971).
21. B. T. STOREY, *Archs. Biochem. Biophys.* **121,** 290 (1967).
22. B. C. PRESSMAN, *J. biol. Chem.* **238,** 401 (1963).
23. H. A. LARDY, D. JOHNSON and W. C. MCMURRAY, *Archs. Biochem. Biophys.* **78,** 587 (1958).
24. M. POE and R. W. ESTABROOK, *Archs. Biochem. Biophys.* **126,** 320 (1968).
25. S. S. KATYARE, P. FATTERPAKER and A. SREENIVASAN, *Archs. Biochem. Biophys.* **144,** 209 (1971).