BBA 45886

OLIGOMYCIN EFFECTS ON LIPOLYSIS AND THE OXIDATIVE METABOLISM OF BROWN FAT CELLS

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(Received August 8th, 1969)

SUMMARY

1. Oligomycin inhibited the respiration of rat brown fat cells incubated in the absence but not in the presence of 10 mM glucose.

2. Oligomycin increased the conversion of glucose to CO_2 in brown fat cells. In white fat cells oligomycin diminished the conversion of glucose to CO_2.

3. *m*-Chloro(carbonyl cyanide)phenylhydrazone doubled respiration by brown fat cells and markedly increased the conversion of glucose to CO_2 over a 2-h period in the presence of glucose. In the absence of glucose, *m*-chloro(carbonyl cyanide)-phenylhydrazone reduced respiration of brown fat cells by over 50 %.

4. Octanoic acid (0.9 mM) increased O_2 consumption of brown fat cells over 10-fold in the presence of glucose and 4-fold in the absence of glucose. Oligomycin had only a small effect on octanoate conversion to CO_2 or octanoate-induced respiration of brown fat cells in the presence of glucose, but in the absence of glucose oligomycin markedly inhibited these parameters.

5. Oligomycin blocked the lipolytic response of both brown and white fat cells to epinephrine and theophylline if the antibiotic was added prior to the lipolytic agents.

6. These data indicate that in the presence of glucose both basal and fatty acid-stimulated respiration in brown fat cells is largely oligomycin insensitive under conditions in which hormonal activation of lipolysis is blocked by oligomycin.

INTRODUCTION

Catecholamines markedly stimulate the respiration of brown adipose tissue[1] and brown fat cells[2] by a mechanism secondary to activation of lipolysis. Since no process with the possible exception of cyclic ion flux has been described in brown fat cells which could utilize the large amounts of energy produced by fatty acid-stimulated respiration it is possible that brown fat cells do not couple respiration to oxidative phosphorylation. The hypothesis that brown fat cells are permanently uncoupled was supported by the failure of attempts to isolate brown fat mitochondria with the ability to catalyze oxidative phosphorylation[3,4] and the difficulty in demonstrating elementary particles in negatively stained brown fat mitochondria[4]. However,

Abbreviation. CCCP, *m*-chloro(carbonyl cyanide)phenylhydrazone

this hypothesis was discredited by the isolation of phosphorylating rat brown adipose tissue mitochondria using defatted albumin[5] and by the stimulation of respiration in brown fat cells of rats[6] and hamsters[7] by classical uncouplers of oxidative phosphorylation. These results have indicated that basal respiration in brown fat cells is at least partly coupled.

REED AND FAIN[8] reported that fatty acid-activated respiration in brown fat cells is a K^+-dependent process. They suggested that catecholamine-stimulated lipolysis liberates free fatty acids which uncouple oxidative phosphorylation. Their results indicated that fatty acids not only provide substrate for oxidation but also act as uncoupling agents in the presence of K^+.

The present experiments were designed to investigate the effects of relatively high concentrations of *m*-chloro(carbonyl cyanide)-phenylhydrazone (CCCP), a potent uncoupling agent, and of oligomycin, an antibiotic which blocks a late step in the transfer of oxidative energy to ATP. The transport of ions across mitochondrial membranes is blocked by classical uncouplers like chloro-carbonyl phenylhydrazones but not by oligomycin[9]. The details of the mechanisms involved remain obscure. In the present report some unexpected effects of oligomycin on the metabolism of brown fat cells are reported.

EXPERIMENTAL PROCEDURE

Dorsal interscapular brown adipose tissue was obtained from 130–160-g female Sprague–Dawley rats (Charles River CD strain) fed laboratory chow *ad libitum*. Brown fat cells were isolated by digestion of fat pads for 1 h with crude bacterial collagenase (*Clostridium histolyticum*, Worthington Biochemical Corporation) at a concentration of 1 mg/ml in phosphate buffer containing 4 % albumin[10]. Brown fat cells obtained for studies reported in Table I and Fig. 1 were isolated in 1 mg/ml collagenase *plus* 1 mg/ml of trypsin. Ovomucoid was present in the incubation medium in those experiments in which cells were isolated by digestion with trypsin to inhibit any traces of trypsin. White fat cells were isolated by the procedure of RODBELL[11] after digestion of parametrial fat pads with 0.5 mg/ml collagenase and 1 mg/ml trypsin. The buffer was made up fresh daily and adjusted to pH 7 4 with NaOH after addition of regular or defatted bovine Fraction V albumin powder (Pentex, lot P-55). Defatted albumin was prepared by the procedure of GUILLORY AND RACKER[5]. The phosphate buffer contained the following: NaCl, 128 mM; $CaCl_2$, 1.4 mM; $MgSO_4$, 1.4 mM, KCl, 5.2 mM; and Na_2HPO_4, 10 mM (pH adjusted to 7.4 with HCl)

O_2 consumption was measured in a Gilson respirometer at 37°. Warburg side-arm respirometer flasks were siliconized prior to each experiment. The respirometer flasks, containing 3 ml of medium, were gassed with 100 % O_2 for 3 min and equilibrated for 30 min prior to the start of the period over which respiration was measured. CO_2 was absorbed by 0.2 ml of 10 % KOH on rolled strips of filter paper (5 mm × 80 mm) in the center well. The basal rate of O_2 consumption was linear over the entire period of the respirometer studies.

In the studies when respiration was not measured, the cells were incubated in 17 mm × 100 mm polyethylene tubes containing a total of 1.5 ml of medium. These tubes were gassed with 100 % O_2 and then capped. The values for each experiment are the average of duplicate flasks and represent the change occurring during the

incubation period over that of initial controls which were incubated for 5 min. CO_2 was collected on rolled filter papers suspended in plastic center wells (Kontes Glass Co.) attached to rubber septum stoppers which were used to seal the tubes At the end of the incubation period 0 2 ml of NCS solubilizer (Nuclear Chicago) was added to the filter paper and 0 25 ml in H_2SO_4 was added to the medium The filter papers were removed after 30 min and counted in 10 ml of toluene–Omnifluor^R solution. The CO_2-containing filter paper rolls from respirometer experiments were counted in 10 ml of BRAY's[12] solution. Total lipid radioactivity *minus* counts in fatty acid was considered to represent the conversion of glucose to glyceride–glycerol and was determined as previously described[11]. The values for the conversion of labeled glucose and octanoate to CO_2 are expressed as μmoles of substrate converted to CO_2 and are minimal estimates. These values actually represent the appearance of radioactivity in CO_2, and no correction was made for dilution of the labeled acetate from octanoate by unlabeled acetate derived from glucose and for the reverse when octanoate was labeled.

At the end of the 4-h incubation period 50-μl aliquots of the medium were taken for glycerol[13] and lactate[14] analysis. Free fatty acids were determined in the cells and remainder of the medium by a modification (hexane substituted for heptane) of the procedure of DOLE AND MEINERTZ[15]. Triglyceride content was based on total fatty acids after saponification. Each experiment was repeated at least 5 times on as many days.

Stock solutions of oligomycin were dissolved in ethanol. A comparable amount of ethanol was added to all control flasks. Octanoic acid neutralized to pH 7 4 with NaOH was added to the flasks and tubes

The sources of the chemicals were as follows: L-epinephrine, oligomycin (15 % oligomycin A, 85 % oligomycin B), and sodium octanoate; Sigma; L-norepinephrine (arterenol) bitartrate and trypsin, Calbiochem; theophylline, Mallinckrodt; *m*-chloro-(carbonyl cyanide)phenylhydrazone, K and K laboratories, Brooklyn, N.Y., and ^{14}C-labeled glucose and octanoic acid, New England Nuclear. Glycerokinase, α-glycerophosphate dehydrogenase and muscle lactate dehydrogenase were obtained from Boehringer Mannheim.

RESULTS

Incubation with oligomycin (3.2 μg/ml) for 15 min prior to the addition of theophylline or norepinephrine abolished their stimulatory effects on lipolysis in both brown and white fat cells (Fig. 1). Oligomycin reduced lipolysis less if added at the same time as these agents, and the smallest reduction of lipolysis was seen if oligomycin was added after the agents. Even when oligomycin was added 15 min after the lipolytic agents, lipolysis due to norepinephrine was reduced by 40 % and that due to theophylline was reduced by 70 % in brown fat cells. Effects of norepinephrine and theophylline on free fatty acid release (not shown) paralleled those on glycerol release shown in Fig. 1.

The effect of oligomycin on glucose metabolism by brown and white fat cells in the same series of experiments shown in Fig. 1 was quite different. Oligomycin inhibited the conversion of glucose to CO_2 in white fat cells while in brown fat cells oligomycin stimulated glucose conversion to CO_2 (Table I). Glucose conversion to

glyceride-glycerol was reduced by oligomycin in both brown and white fat cells (Table I).

The addition of fatty acid (sodium octanoate, 0.7 mM) to brown fat cells in the presence of 2.8 mM glucose resulted in changes in glucose conversion to CO_2 and glyceride-glycerol which were similar to those produced by oligomycin (Table I) In

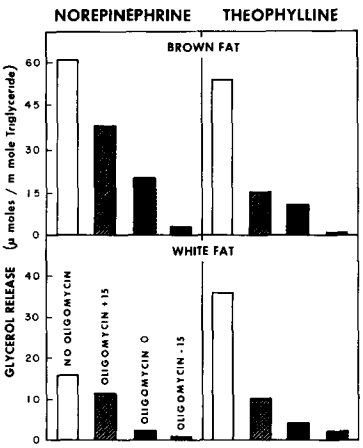

Fig 1. Oligomycin inhibition of lipolysis Brown fat cells (12 μmoles triglyceride), top half of figure, or white fat cells (32 μmoles triglyceride), bottom half of figure, were incubated for 4 h in 1 5 ml of medium containing 2 8 mM glucose and 0 1 mg/ml of ovomucoid The values shown are for lipolysis in the presence of 0 033 μg/ml of norepinephrine (left side of figure) or 1 4 mM theophylline (right side of figure) Glycerol release in the absence of added lipolytic agents was less than 1 μmole of glycerol per mmole of triglyceride for both brown and white fat cells The open bars represent flasks incubated with lipolytic agents alone while striped bars represent flasks in which 3 2 μg/ml of oligomycin was added 15 min after the lipolytic agents The closed bars represent flasks in which oligomycin was added 15 min before the lipolytic agents and stippled bars the addition of oligomycin with the lipolytic agents The values are the means of 5 paired replications done on separate days

TABLE I

A COMPARISON OF THE EFFECTS OF OLIGOMYCIN AND OCTANOIC ACID ON GLUCOSE METABOLISM

Brown (12 4 μmoles of triglyceride per flask) and white (32 μmoles of triglyceride) fat cells from fed rats were isolated in 1 mg/ml collagenase *plus* 0 5 mg/ml trypsin for 1 h The cells were incubated for 4 h in 1 5 ml of 4% albumin buffer containing 2 8 mM uniformly labeled [^{14}C]glucose and 0 1 mg/ml of ovomucoid. The conversion of glucose to CO_2 and glyceride-glycerol is based on the μmoles of labeled glucose converted to these products per mmole of triglyceride The concentration of oligomycin was 3 2 μg/ml and of octanoate was 0 7 mM The basal values are the means of 5 paired replication ± standard error The increments are shown as the mean ± standard error of the paired differences

	Basal	*Increment due to oligomycin*	*Increment due to sodium octanoate*
Brown fat cells			
Glucose to CO_2	2 60 ± 0 18	+4 63 ± 0 97	+2 49 ± 0 20
Glucose to glyceride-glycerol	1 26 ± 0 04	−0 19 ± 0 07	−0 26 ± 0 03
White fat cells			
Glucose to CO_2	1.51 ± 0 23	−1 05 ± 0 15	−0 80 ± 0 23
Glucose to glyceride-glycerol	1 70 ± 0 20	−1 11 ± 0 13	+0 67 ± 0 14

white fat cells both oligomycin and octanoate decreased glucose conversion to CO_2 while glyceride-glycerol formation from glucose was decreased by oligomycin and stimulated by octanoate

In the studies shown in Fig. 1 and Table I, the brown and white adipose tissues were digested with trypsin and collagenase to abolish any effects of insulin or insulin-like substances in the albumin buffer on the action of oligomycin, octanoate, or lipolytic agents. Digestion of brown or white adipose tissue with collagenase in the presence of trypsin selectively inactivates the ability of these cells to respond to insulin but not to lipolytic agents[16]. Trypsin treatment did not affect the stimulation of brown fat cell respiration by added fatty acids[16].

TABLE II

STIMULATION OF LACTATE FORMATION BY OLIGOMYCIN IN BROWN AND WHITE FAT CELLS

Brown (5 6 μmoles of triglyceride per flask) and white (32 μmoles of triglyceride) fat cells from fed rats were incubated for 4 h in 1 5 ml of 4% albumin buffer containing 2 8 mM uniformly labeled [^{14}C]glucose The conversion of glucose to CO_2 is based on the μmoles of labeled glucose converted to these products per mmole of triglyceride The values for lactate were based on an enzymatic assay The concentration of oligomycin was 3 2 μg/ml and of insulin was 0 16 munit/ml The basal values are the means of 5 paired replications ± standard error for white fat cells and 7 for brown fat cells The increments due to oligomycin are shown as the mean ± standard error of the paired differences

	Basal	*Increment due to oligomycin*
Brown fat cells		
Glucose to CO_2		
Without insulin	3 0 ± 0.2	+1.8 ± 0.5
+ insulin	20.2 ± 3.0	+7 5 ± 1 4
Glucose to lactate		
Without insulin	5.6 ± 1 1	+2 2 ± 0 6
+ insulin	21 3 ± 3 0	+8 2 ± 3.0
White fat cells		
Glucose to CO_2		
Without insulin	5 3 ± 0 3	−2 5 ± 0 7
+ insulin	30 0 ± 4 8	−3.0 ± 1 0
Glucose to lactate		
Without insulin	1.4 ± 0.3	+0.5 ± 0 1
+ insulin	1.9 ± 0 5	+1 3 ± 0.5

The effects of oligomycin on glucose conversion to CO_2 were also seen in cells isolated by digestion with collagenase in the absence of trypsin and incubated either without or with insulin (Table II). In contrast to the opposite effects of oligomycin on glucose conversion to CO_2 in brown and white fat cells the formation of lactate was increased in both types of cells by oligomycin. In slices of rat brain[17] or kidney[18] oligomycin markedly increases lactate formation under conditions where respiration is inhibited by less than 30%.

In view of the finding (Tables I and II) that oligomycin stimulated the oxidation of glucose by brown fat cells, it was not surprising that oligomycin failed to inhibit respiration of brown fat cells incubated for 2.5 h in the presence of 10 mM glucose (Fig. 2).

CCCP, a potent uncoupler of oxidative phosphorylation, doubled respiration of brown fat cells incubated in the presence of 10 mM glucose and markedly increased the conversion of labeled glucose to CO_2 (Fig. 2) However, in the absence of glucose oligomycin and CCCP inhibited respiration by more than 50 %. The basal rate of respiration was unaffected by the omission of glucose from the medium (Fig. 2)

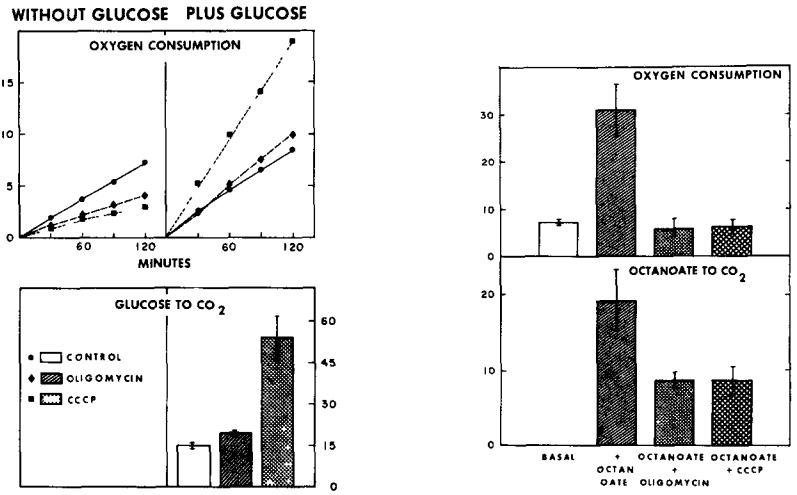

Fig 2 The effects of oligomycin and CCCP on the respiration of brown fat cells in the presence and absence of 10 mM glucose Brown fat cells (8 μmoles triglyceride per flask) from fed rats were incubated at 37° for 2 5 h in 3 ml of 4 % albumin (defatted) buffer with or without randomly labeled [^{14}C]glucose The basal or control values are indicated by open bars and closed circles The values in the presence of oligomycin (3.2 μg/ml) are represented by striped bars and closed diamonds while those for CCCP (50 μM) are shown by stippled bars and closed squares The agents were added at the start of the 2 5-h incubation period while O_2 consumption was monitored over the final 2 h of the incubation period The conversion of labeled glucose to CO_2 in μmoles glucose converted per mmole of triglyceride over 2 5 h is the mean ± standard error of 5 experiments O_2 consumption is expressed as μl O_2/μmole triglyceride for the same experiments

Fig 3 Inhibition by oligomycin (3.2 μg/ml) and CCCP (50 μM) of O_2 consumption by brown fat cells isolated and incubated in the absence of glucose. Free brown fat cells (8 μmoles triglyceride per flask) from fed rats were incubated for 2 5 h in 3 ml of 4 % albumin (defatted) without added glucose Oligomycin and CCCP were added at the start of the incubation period while [1-^{14}C]-octanoate (0 9 mM) was added 20 min after the start of the incubation period O_2 consumption was monitored during the final 2 h of the incubation period and is expressed in μl/mmole of triglyceride Octanoate conversion to CO_2 is in μmoles/mmole of triglyceride The values are the mean ± standard error of 5 paired experiments.

Octanoic acid addition to brown fat cells produced only a 4-fold increase in respiration in the absence of glucose (Fig. 3). Addition of oligomycin or CCCP 20 min before that of octanoic acid to cells without glucose blocked the increase in respiration due to the fatty acid (Fig. 3). Similarly, both oligomycin and CCCP inhibited the conversion of octanoic acid to CO_2 by at least 50 % in the absence of glucose.

The addition of octanoic acid to brown fat cells incubated in the presence of 10 mM glucose caused a 3-fold increase in the conversion of labeled glucose to CO_2 as well as marked stimulation of respiration (Table III). When oligomycin was added before octanoic acid, O_2 uptake was inhibited by about 25 %; addition of the antibiotic after octanoic acid resulted in a slightly smaller inhibition. Oligomycin, when

TABLE III

EFFECTS OF OLIGOMYCIN (3 2 μg/ml) AND CCCP (50 μM) ON O_2 CONSUMPTION AND THE CONVERSION OF OCTANOIC ACID AND GLUCOSE TO CO_2 IN BROWN FAT CELLS

Free brown fat cells (8 μmoles of triglyceride per flask) were incubated for 2 5 h in 3 ml of 4 % albumin (defatted) buffer containing 10 mM glucose with or without 0 9 mM octanoic acid O_2 consumption was monitored during the last 2 h of the incubation period The conversion of randomly labeled [^{14}C]glucose and [1-^{14}C]octanoate to CO_2 is expressed as μmoles of glucose or octanoate converted to CO_2 per mmole of triglyceride 2 replicates were run for each condition, one in which the glucose was labeled and the other in which the octanoate was labeled O_2 consumption is expressed as μl of O_2 consumed per μmole of triglyceride Octanoic acid was added at the start of the experiment except when oligomycin or CCCP was added before the fatty acid The values are the means of 5 paired experiments and the increments due to oligomycin and CCCP added 20 min before or 20 min after octanoic acid are the mean ± standard error of the paired differences

	Basal	*Plus octanoic acid*	*Increment due to*			
			Oligomycin added 20 min prior to octanoate	*Oligomycin added 20 min after octanoate*	*CCCP added 20 min prior to octanoate*	*CCCP added 20 min after octanoate*
O_2 consumption	7 4	81 8	−20 0 ± 5 4	−16 9 ± 4 9	−61 6 ± 8 2	−35 1 ± 4 1
Conversion of glucose to CO_2	14 7	44 7	−19 8 ± 5 1	−15 6 ± 3 4	− 1 2 ± 6 0	− 2 1 ± 2 2
Conversion of octanoate to CO_2		62 0	−20 1 ± 2 6	− 7 1 ± 2 1	−35 8 ± 3 5	−13 2 ± 6 4

present prior to the fatty acid, inhibited the conversion of glucose to CO_2 nearly 50 %, and again the effect was only slightly less when oligomycin was added after octanoic acid. In contrast, the conversion of octanoic acid to CO_2 was inhibited to a much greater extent if the antibiotic was added before rather than after the fatty acid, this inhibition was about 30 % and 10 %, respectively, and the difference was significant.

The effects of CCCP were more marked than those of oligomycin (Table III). Addition of the uncoupler 20 min before the octanoic acid inhibited nearly 75 % of the respiration of brown fat cells; addition of CCCP after the fatty acid caused significantly less inhibition, in contrast to the results with oligomycin. The conversion of glucose to CO_2 was unaffected by CCCP regardless of the time of addition. CCCP alone increased basal glucose oxidation to CO_2 (Fig. 2) to approximately the same extent as octanoic acid.

DISCUSSION

Previously it was found that the addition of oligomycin 4 min before that of norepinephrine abolished the early stimulatory effect of norepinephrine on respiration of brown fat cells incubated without glucose[2]. Addition of oligomycin after norepinephrine did not abolish the stimulatory effect of norepinephrine on respiration during the next 3 min[2]. PRUSINER *et al.*[7,19] have also investigated the early effects of fatty acids, catecholamines uncouplers and oligomycin on the oxidative metabolism of fat cells isolated from the brown fat of starved hamsters. They found that in hamster

brown fat cells incubated without glucose the addition of oligomycin prior to or several minutes after catecholamines blocked the stimulation of respiration due to these agents[7]. The present results indicate that the activation of lipolysis by catecholamines or methyl xanthines occurs by an oligomycin-sensitive process while the activation of respiration by added free fatty acids is largely insensitive to oligomycin.

The assumption that ATP is required for activation of lipolysis in brown fat cells is based on the finding that incubation of cells with CCCP blocked the lipolytic action of dibutyryl-3',5'-AMP (ref. 6) FASSINA[20] has also observed that oligomycin blocked the activation of lipolysis by dibutyryl-3',5'-AMP in white fat cells. The present studies are in agreement with these findings since oligomycin blocked the action of lipolytic agents in both brown and white fat cells.

The most striking finding in these studies was the failure of oligomycin to inhibit the respiration of brown fat cells incubated in the presence of glucose despite the fact that it was able to block the hormonal activation of lipolysis under these conditions. If glucose was not present in the medium, respiration was inhibited by both oligomycin and CCCP (Fig. 2) In the absence of glucose the formation of ATP from glycolysis may be insufficient to activate substrate for oxidation. This might account for the inhibition of basal and fatty acid-activated respiration by oligomycin and CCCP in brown fat cells incubated without added glucose. The same interpretation could be given for the failure of fatty acid even in the presence of glucose to stimulate respiration in brown fat cells incubated with the uncoupler prior to addition of fatty acid. Under these conditions there may be no extra ATP which can be used for activation of the fatty acids prior to oxidation.

Oligomycin inhibits both respiration and phosphorylation of isolated liver mitochondria but only phosphorylation by sub-mitochondrial particles[21]. Oligomycin seldom inhibits the respiration of whole cell preparations by more than 30%[17,18,22] except for tumor and tissue-culture cells with high basal rates of aerobic glycolysis[23]. HEPP *et al.*[24] reported that oligomycin inhibited the respiration of white fat cells incubated in the presence of both glucose and insulin by about 50% while the stimulation of respiration by catecholamines was unaffected. The respiration of rat-liver slices[22] is inhibited by less than 25% in the presence of oligomycin while that of isolated rat-liver mitochondria is almost completely blocked by oligomycin[21]. These studies suggest that in intact mammalian cells ADP competes with other processes for the conserved energy derived from electron transport. If the phosphorylation of ADP is blocked by oligomycin then this energy is either directly utilized for energy-requiring reactions such as ion transport or is dissipated. In a sense it appears that intact cells resemble submitochondrial particles[21] which loosely couple phosphorylation to electron transport. This does not necessarily mean that the P/O ratio of intact cells is less than three, but only that in the intact cell the energy which ordinarily is utilized for ATP formation from ADP can be largely dissipated or directly utilized in the presence of oligomycin.

The inability of oligomycin to inhibit basal respiration by brown fat cells in the presence of glucose should not be interpreted to mean that basal respiration is dissociated from ATP formation. The failure of lipolytic agents to activate lipolysis in the presence of oligomycin and the complete inhibition of fatty acid-stimulated respiration by oligomycin in the absence of glucose suggest that ATP is synthesized by brown fat mitochondria under basal conditions. Our results indicate only that

there is no obligatory linkage between respiration and ATP formation in brown fat cells which is probably true of most cells. The extent to which oligomycin inhibits respiration of intact cells may represent only the degree to which cells are able to maintain sufficient ATP concentrations from substrate phosphorylation for activation of compounds prior to oxidation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Research Grant AM-10149 from the National Institute of Arthritis and Metabolic Diseases.

S.C.L. is a Predoctoral Fellow of the United States Public Health Service.

M.P.C. is a predoctoral trainee (TI-CA 05007) of the United States Public Health Service and is supported by an institutional grant from the American Cancer Society.

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