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Effects of Menadione and Vitamin K_5 on Glucose Metabolism, Respiration, Lipolysis, Cyclic 3′,5′-Adenylic Acid Accumulation, and Adenyl Cyclase in White Fat Cells

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SUMMARY

The addition of menadione (2-methyl-1,4-naphthoquinone) to white fat cells in the presence of catecholamines and theophylline markedly increased the accumulation of cyclic AMP within 5 min. However, menadione inhibited the activation of adenyl cyclase by norepinephrine in fat cell "ghosts." Vitamin K_5 (2-methyl-4-amino-1-naphthol) actually inhibited cyclic AMP accumulation except under conditions in which autoxidation of K_5 occurred. The products obtained from air oxidation of vitamin K_5 were able to increase the cyclic AMP content of white fat cells in the presence of catecholamines and theophylline. The increase in cyclic AMP content due to menadione was largely intracellular and did not involve any alteration in the total ATP content of fat cells. Both menadione and vitamin K_5 (5 μg/ml) increased respiration, glucose oxidation, lactate accumulation, and fatty acid synthesis. The stimulation of respiration due to vitamin K_5 was potentiated by oligomycin, while that due to menadione was inhibited. The increases in oxygen consumption, lactate accumulation, glucose oxidation, and fatty acid biosynthesis produced by both menadione and vitamin K_5 were sensitive to cyanide. Antimycin A preferentially inhibited the increases in glucose metabolism and respiration due to menadione but had a smaller effect on those attributable to vitamin K_5. The insulin-like effects of these drugs could be distinguished from the action of insulin itself by the greater stimulation of fatty acid synthesis induced by insulin. Vitamin K_5, like insulin, inhibited the lipolytic action of catecholamines in the absence of theophylline after a lag period of 5 min, while in the presence of 1 mM theophylline the antilipolytic action was largely overcome. Insulin did not affect the accumulation of cyclic AMP, whereas vitamin K_5 reduced cyclic AMP accumulation under conditions in which these agents had equivalent effects on lipolysis. These results indicate that certain naphthoquinones can mimic the action of insulin on lipolysis and glucose metabolism while increasing cyclic AMP accumulation.

INTRODUCTION

A number of derivatives of vitamin K_1, such as 2-methyl-1,4-naphthoquinone (menadione or vitamin K_3) and 2-methyl-4-amino-1-naphthol hydrochloride (vitamin K_5), are effective when given orally (1). However, the administration of large amounts of these compounds to infants has been associated with hemolysis resulting in hyperbilirubinemia. The hemolytic effects of menadione and vitamin K_5 have been

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attributed to depletion of reduced glutathione in red blood cells secondary to oxidation of human hemoglobin to methemoglobin by menadione (1, 2).

The role of vitamin K compounds in the formation of hepatic factors involved in blood clotting is well established, but the possibility that these compounds might function as oxidation-reduction carriers in intact cells has not been demonstrated in mammals (3). The present investigation was prompted by reports that vitamin K_5 was capable of stimulating the accumulation of cyclic AMP and the metabolism of glucose and of inhibiting lipolysis in white fat cells (4, 5). It has been found that it is not vitamin K_5 which affects cyclic AMP accumulation in fat cells, but the quinones produced as a result of oxidation of vitamin K_5 .

METHODS

Free white fat cells were obtained from 120–160-g female Sprague-Dawley rats (Charles River CD strain) fed laboratory chow ad libitum. White fat cells were isolated by a modification of the procedure of Rodbell (6) from the pooled parametrial adipose tissue of three or more rats. Krebs-Ringer-phosphate buffer of the following composition was used in all experiments: NaCl, 128 mM; $CaCl_2$, 1.4 mM; $MgSO_4$, 1.4 mM; KCl, 5.2 mM; and Na_2HPO_4 , 10 mM. The buffer was prepared daily and adjusted to pH 7.4 with NaOH after addition of Armour bovine fraction V albumin powder or Pentex fraction V albumin defatted by the procedure of Guillory and Racker (7).

For studies in which the formation of labeled cyclic AMP was to be examined, the cells were washed once and then incubated for 15 min in 4% albumin buffer containing adenine-8-^{3}H (23 Ci/mmole). The labeled cells were washed once with buffer and then added to incubation flasks.

In studies in which ATP and glucose 6-phosphate were to be determined, the reactions were stopped by addition of 8% (v/v) perchloric acid in 40% (v/v) ethanol. ATP and glucose 6-phosphate were determined in aliquots of the neutralized filtrates by fluorometric procedures of Williamson and Corkey (8). Labeled cyclic AMP was determined in neutralized perchloric acid filtrates by adding 0.2 ml of 5% zinc sulfate and 0.1 ml of saturated sodium hydroxide and adjusting the pH to between 7 and 8. The mixture was centrifuged, and the procedure was repeated. In other studies the medium or cells were boiled for 1 min prior to the barium-zinc procedure. The barium-zinc filtrate was chromatographed on a 4 × 70 mm column of Dowex 50 (AG50W-X8, 100–200 mesh), and the cyclic AMP fraction was collected, lyophilized, and then resuspended in 0.1 ml of 50 mM Tris-HCl (pH 7.0). One 25-μl sample was removed for determination of labeled cyclic AMP and counted in a liquid scintillation spectrometer using Bray's solution (9). Total cyclic AMP was determined on other aliquots by the procedure of Goldberg *et al.* (10) as described by Fain, Dodd, and Novak (11).

Adenyl cyclase activity was determined using fat cell ghosts prepared by hypotonic lysis of white fat cells by the procedure of Birnbaumer *et al.* (12). The ghosts were used immediately and incubated at 37° for 20 min in a total volume of 100 μl containing 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 30 mM KCl, 1 mM cyclic AMP, 8 mM phosphoenolpyruvate, 10 μg of pyruvate kinase, 1 mM ATP, and 0.2 μCi of tritiated ATP (New England Nuclear Corporation). At the end of the incubation period the tubes were boiled for 3.5 min after the addition of 50 μl of 5 mM cyclic AMP. The total volume was added to a 3 × 65 mm column of Dowex AG50W-X8, 100–200 mesh. The fraction containing cyclic AMP was collected, and 0.2 ml of 5% zinc sulfate and 0.1 ml of saturated barium hydroxide were added. The pH was adjusted to 7–8. Following centrifugation the procedure was repeated, and 1 ml was counted in 10 ml of Bray's scintillation mixture (9) in a liquid scintillation spectrometer. Protein concentration was determined according to the procedure of Lowry *et al.* (13).

Oxygen consumption was measured in a Gilson respirometer with air as the gas phase at 37°. The single side-arm Warburg respirometer flasks were treated with silicone prior to each experiment. The flasks, containing the cells in 3 ml of medium, were equilibrated for 30 min prior to the start of

the 2-hr period over which respiration was measured. Carbon dioxide was absorbed by rolled strips of filter paper (5 x 80 mm) in the center wells, to which 0.2 ml of 10% KOH had been added. When labeled glucose was present, the strips of paper were removed at the end of the experiment and counted in Bray's solution (9).

The conversion of labeled glucose to carbon dioxide, glyceride-glycerol, and fatty acids was determined by the procedures of Rodbell (6). The amount of cells added to each flask was calculated after determination of the total fatty acid content following saponification and assuming 3 mmoles of fatty acid per gram of cells (6). Aliquots of the medium were removed for analysis of glycerol (14) and lactate (15) at the end of the incubation.

Menadione (2-methyl-1,4-naphthoquinone), crystalline antimycin A from *Streptomyces kitazawaensis* mycelia, and oligomycin (15% A and 85% B) were obtained through Sigma Chemical Company; stock solutions were prepared in 95% ethanol. In all experiments with these agents an equivalent amount of ethanol was added to the control flasks, although the addition of up to 10 μl of alcohol per flask has produced no detectable effect on the metabolism of brown or white fat cells. Solutions of vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride), obtained from Nutritional Biochemicals Corporation, were prepared fresh each day. Oxidized vitamin K_5 was prepared by air oxidation of vitamin K_5 dissolved in water. The oxidized product was insoluble in water and obtained by filtration of the reaction mixture followed by washing with water and drying of the deep red product in a desiccator. The insulin was a glucagon-free porcine preparation donated by Eli Lilly and Company.

RESULTS

After incubation of white fat cells for 30 min in the presence of norepinephrine and theophylline, there was an increase due to vitamin K_5 in the accumulation of both total and labeled cyclic AMP (Table 1). Fluoride is a potent activator of adenyl cyclase in broken-cell preparations (12). In intact fat cells fluoride inhibited cyclic AMP accumulation and lipolysis in the presence of norepinephrine and theophylline (Table 1). In this experiment an appreciable amount of cyclic AMP was present after 30

TABLE 1

Effect of vitamin K_5 and fluoride on lipolysis and cyclic AMP accumulation

White fat cells were isolated by digestion of parametrial adipose tissue for 45 min with 1 mg/ml of collagenase. The cells were washed, incubated with adenine-8-^{3}H for 15 min, and then washed to remove unincorporated adenine. White fat cells (50 mg/tube) were incubated for 5 or 30 min in 1.5 ml of buffer containing 2% defatted Pentex albumin. The concentration of theophylline was 1 mM; norepinephrine, 1.3 μM; vitamin K_5, 5 μg/ml; and sodium fluoride, 10 mM.

Additions	Cyclic AMP accumulation				Glycerol release (30 min)
	Total		Labeled		
	5 min	30 min	5 min	30 min	
	nmoles/g		*cpm*		*μmoles/g*
None	0	0.1	7	5	1.0
Norepinephrine + theophylline	33.4	12.5	475	225	21.4
Vitamin K_5	2.3	0.7	75	20	2.6
Vitamin K_5 + norepinephrine + theophylline	43.3	42.0	1065	610	17.5
Sodium fluoride		0		0	0.6
Fluoride + norepinephrine + theophylline		1.8		27	10.1
Fluoride + vitamin K_5 + norepinephrine + theophylline		3.6		50	10.5

min of incubation. However, in the experiments shown in Figs. 1 and 2, the amount of cyclic AMP that accumulated in 30 min was always less than 10% of that seen after 5 min of incubation with any of the agents tested. Vitamin K_5 was relatively ineffective against the lipolytic action of norepinephrine in the presence of theophylline (Table 1).

In the next experiments I compared the effects of insulin and vitamin K_5 on lipolysis, ATP, and cyclic AMP in the presence of norepinephrine either without or with methylxanthines (Fig. 1). Both insulin and vitamin K_5 reduced by two-thirds the lipolytic action of norepinephrine alone, but in the presence of theophylline their antilipolytic action was largely overcome. Despite the similar antilipolytic actions of insulin and vitamin K_5, insulin alone had no effect on cyclic AMP accumulation whereas vitamin K_5 increased the accumulation of

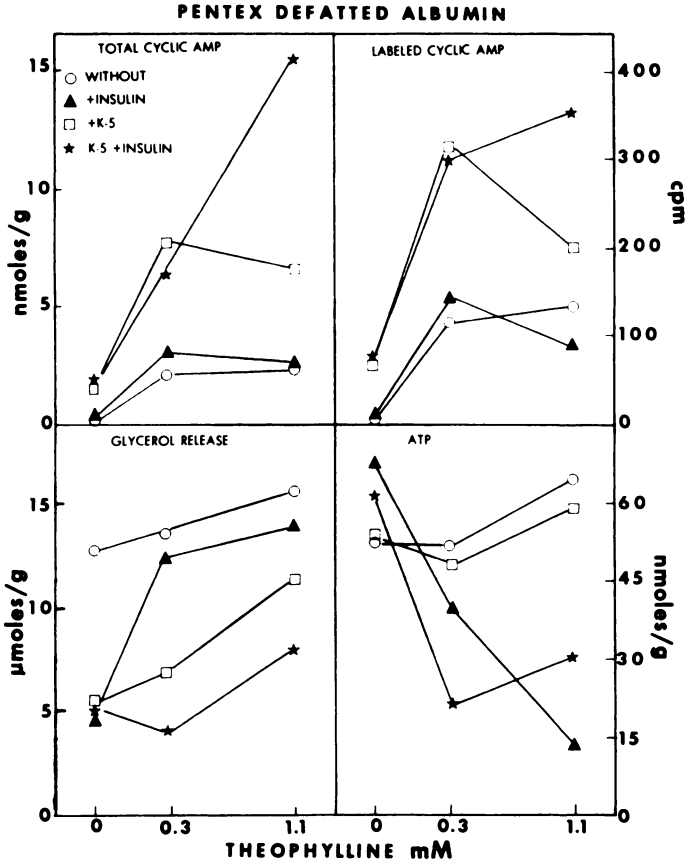

FIG. 1. *Stimulation by vitamin K_5 of cyclic AMP accumulation by fat cells incubated in Pentex albumin containing norepinephrine*

White fat cells were isolated by digestion of adipose tissue for 45 min with 1 mg/ml of collagenase. The cells were washed, incubated with adenine-8-^{3}H for 15 min, and then washed to remove unincorporated adenine. Fat cells (80 mg/tube) were incubated for 5 or 30 min in 1.5 ml of glucose-free buffer containing 4% defatted Pentex albumin. The values are the averages from two paired experiments and represent the increments over zero-time controls except for ATP, which represents total ATP content. Norepinephrine (1.3 μM) was present in all tubes in either the absence or presence of 0.3 or 1.1 mM theophylline. ○, norepinephrine in the absence of insulin or vitamin K_5; ▲, 0.17 milliunit/ml of insulin; □, 5 μg/ml of vitamin K_5; ★, insulin plus vitamin K_5. Glycerol release was measured at 30 min, while ATP and total and labeled cyclic AMP are 5-min values. There was no detectable glycerol release in the absence of norepinephrine in these experiments.

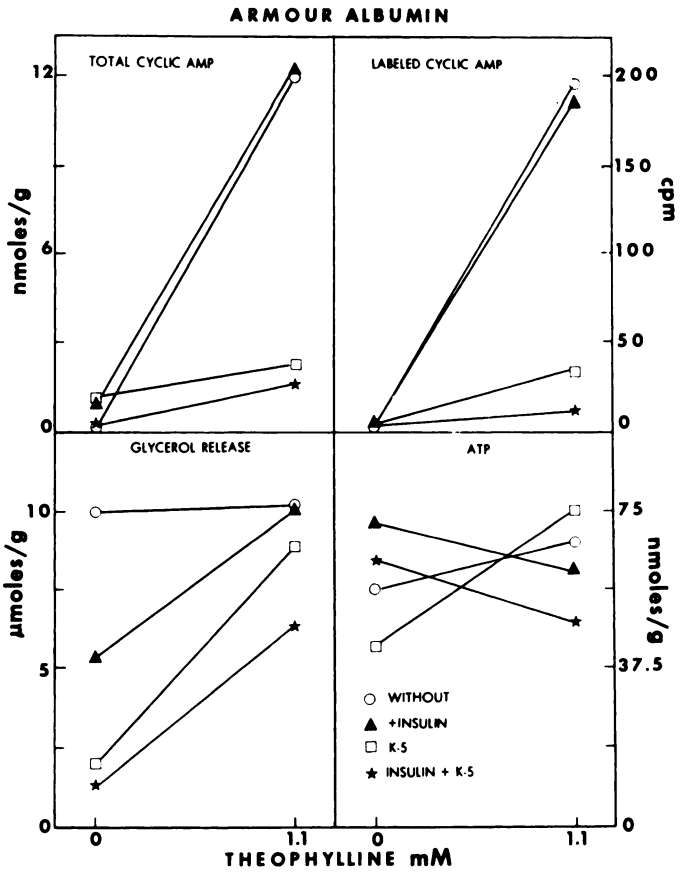

FIG. 2. *Inhibition by vitamin K_5 of cyclic AMP accumulation by fat cells incubated in Armour albumin containing norepinephrine*

White fat cells were isolated by digestion of adipose tissue for 45 min with 1 mg/ml of collagenase. The cells were washed, incubated with adenine-8-^{3}H for 15 min, and then washed to remove unincorporated adenine. Fat cells (100 mg/tube) were incubated for 5 or 30 min in 1.5 ml of glucose-free buffer containing 4% Armour albumin. The values are the averages from two paired experiments and represent the increments over zero-time controls except in the case of ATP content. Norepinephrine (1.3 μM) was present in all tubes in either the absence or presence of 1.1 mM theophylline. ○, norepinephrine in the absence of insulin or vitamin K_5; ▲, 0.17 milliunit/ml of insulin; □, 5 μg/ml of vitamin K_5; ★, insulin plus vitamin K_5. Glycerol release was measured at 30 min, while ATP and total and labeled cyclic AMP are 5-min values. The lines between 0 and 1.1 mM theophylline for each group are drawn for convenience and are not meant to imply that a linear relationship exists at intermediate points.

both total and labeled cyclic AMP. Epinephrine alone produced nearly maximal stimulation of lipolysis, but the amount of cyclic AMP found with norepinephrine alone was less than 2% of that seen with norepinephrine in the presence of 1.1 mM theophylline. The large accumulation of cyclic AMP seen in the presence of lipolytic agents and methylxanthines may represent an "overshoot" phenomenon not associated with any physiological function. The data in Fig. 1 indicate that there was no correlation between cyclic AMP accumulation at 5 min and lipolysis over a 30-min incubation period.

Neither vitamin K_5 nor theophylline affected ATP content at 5 min (Fig. 1) or at 30 min (data not shown). There was an increase in ATP due to insulin in the presence of norepinephrine alone, which was

changed to a decrease in the presence of theophylline in both the presence and absence of vitamin K_5 and was observed in both experiments.

In the studies shown in Table 1 and Fig. 1 the cells were incubated in defatted Pentex albumin. Although vitamin K_5 increased glucose oxidation by fat cells incubated in Armour albumin (Table 2), it inhibited the cyclic AMP accumula-

TABLE 2

Stimulation by menadione of cyclic AMP accumulation by fat cells in Armour albumin

White fat cells were isolated by digestion for 45 min with 1 mg/ml of collagenase. The cells were washed, and half of them were incubated for 15 min with adenine-8-^{3}H and then washed to remove unincorporated adenine. The fat cells which had been incubated with labeled adenine were then incubated for 5 min in 1.5 ml of buffer containing 2.8 mM glucose and 2% Armour albumin plus 1.3 μM norepinephrine and 1 mM theophylline. The other half of the cells (70 mg/tube) were incubated for 45 min in 1.5 ml of buffer containing 2.8 mM uniformly labeled glucose-^{14}C and 2% Armour albumin.

Addition	Glucose conversion to CO_2	Glucose conversion to fatty acid	Cyclic AMP accumulation	
			Total	Labeled
	μmoles/g	*μmoles/g*	*nmoles/g*	*cpm*
None	0.055	0.04	12	400
Vitamin K_5 (5 μg/ml)	0.75	0.49	12	370
Menadione (5 μg/ml)	0.275	0.12	22	760
Oxidized vitamin K_5 (5 μg/ml)	0.15	0.07	24	750

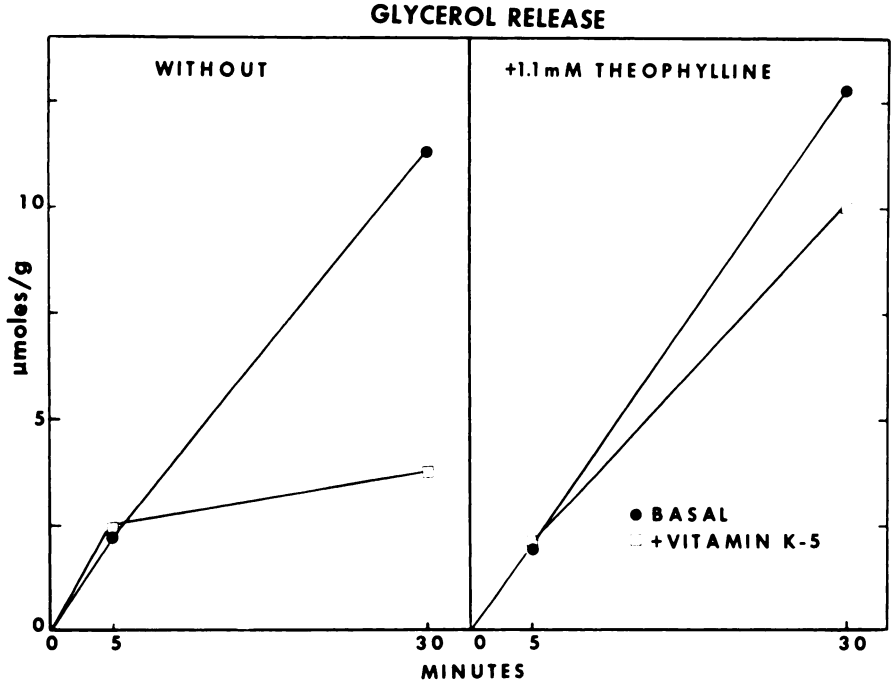

FIG. 3. *Effect of vitamin K_5 on lipolysis*

The values are taken from the experiments shown in Figs. 1 and 2 and are the means of four experiments. The 30-min values are also shown in Figs. 1 and 2. ●, cells incubated with norepinephrine in the absence of vitamin K_5; □, those incubated with 5 μg/ml of vitamin K_5.

tion of cells under the same conditions (Fig. 2). However, vitamin K_5 had the same antilipolytic action as in the experiments shown in Fig. 1. Insulin also had an antilipolytic action, which was overcome in the presence of 1.1 mM theophylline, but insulin had no significant effect on cyclic AMP content. In the experiments with Armour albumin none of these agents had a clear effect on ATP content at 5 min (Fig. 2).

The results in Fig. 3 indicate that the antilipolytic action of vitamin K_5 was delayed in onset and was apparent only after 5 min. The results were pooled from the four experiments shown in Figs. 1 and 2, since there was no difference among the different lots of albumin with respect to the antilipolytic action of vitamin K_5.

The finding that vitamin K_5 increased cyclic AMP accumulation only if cells were incubated in Pentex albumin suggested that not vitamin K_5 but a product of its further metabolism might be responsible. I prepared stock solutions of vitamin K_5 in buffer containing either 4% Pentex or Armour albumin and noted that a red color gradually appeared in the solution containing Pentex albumin but not in that with Armour albumin. If vitamin K_5 was dissolved in water and mixed vigorously, a dark red color appeared. These results suggested that Armour albumin contains a substance which inhibits the autoxidation of K_5 to a quinone or semiquinone. Because complete oxidation of vitamin K_5 (2-methyl-4-amino-1-naphthol) should lead to the formation of menadione (2-methyl-1,4-naphthoquinone), it was of interest to compare the effects of menadione (a pale yellow compound) with those of vitamin K_5 and the red product obtained by its oxidation in air.

In the studies shown in Table 2, fat cells incubated in Armour albumin. Menadione or oxidized vitamin K_5, but not unoxidized K_5, increased cyclic AMP accumulation after 5 min of incubation in the presence of norepinephrine and theophylline. All compounds increased glucose oxidation and conversion to fatty acids.

The increase in cyclic AMP produced by menadione was not the result of a large efflux of cyclic AMP from fat cells. The studies in Fig. 4 indicate that the effect of menadione is exerted largely on the intracellular accumulation of cyclic AMP. The action of menadione was rapid, and was even greater at 5 min than at 15 min when

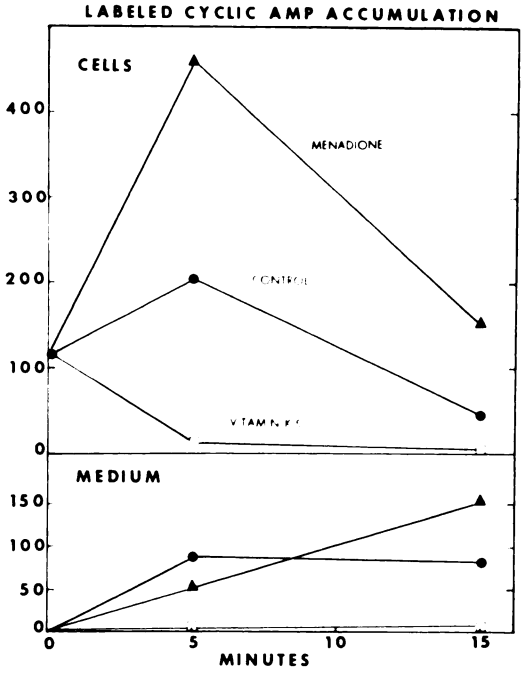

FIG. 4. *Comparison of effects of menadione (vitamin K_3) with those of vitamin K_5 on labeled cyclic AMP accumulation in fat cells*

White fat cells were isolated by digestion of adipose tissue for 45 min with trypsin and collagenase, 0.5 mg/ml each. The cells were washed, incubated with adenine-8-^{3}H for 15 min, and then washed again to remove unincorporated adenine. White fat cells (134 mg/tube) were incubated for 5 or 15 min in 1.5 ml of buffer containing 2% Armour albumin, 1 mM theophylline, and 1.3 μM norepinephrine. The zero-time values are for tubes in which the reactions were stopped within 30 sec after the cells had been added to the water bath. The values for the accumulation of labeled cyclic AMP, in counts per minute, are the means of three paired experiments without vitamin K_5 (●) with 5 μg/ml of vitamin K_5 (□), or with 5 μg/ml of menadione (▲).

the cyclic AMP values had fallen from the peak reached after 5 min. In studies shown in Fig. 4 there was a high zero-time value for intracellular cyclic AMP, since these cells were also incubated with norepinephrine and theophylline.

The marked potentiation of cyclic AMP accumulation in intact cells by menadione in the presence of lipolytic agents was in contrast to its effect on the adenyl cyclase activity of ghosts. Menadione inhibited the increase in adenyl cyclase activity due to saturating concentrations of norepinephrine, without affecting basal adenyl cyclase activity (Table 3).

Since the stimulation of cyclic AMP accumulation by menadione was apparently not attributable to any direct stimulation

TABLE 3

Inhibition of adenyl cyclase activity of white fat cell ghosts by menadione

The adenyl cyclase activity of white fat cell ghosts (104 μg of protein per tube in experiment I and 70 in experiment II) was determined in the absence and presence of norepinephrine (0.2 mM).

Menadione concentration	Experiment I		Experiment II	
	Without norepinephrine	With norepinephrine	Without norepinephrine	With norepinephrine
μg/ml	*nmoles cyclic AMP formed/mg protein/20 min*			
0	2.4	14.4	0.5	17.2
2		0	0.5	14.2
5	1.9	4.8	0.5	14.2
10	1.6	5.1	0	

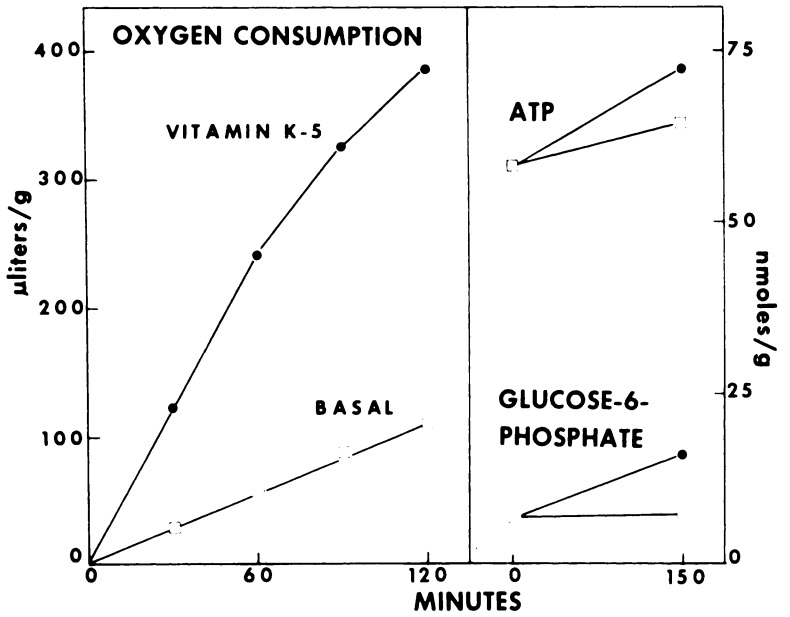

FIG. 5. *Effect of vitamin K_5 on ATP and glucose 6-phosphate content of white fat cells*

Parametrial adipose tissue was digested for 45 min in 4% albumin buffer containing 2.8 mM glucose and collagenase and trypsin, 0.5 mg/ml each. Approximately 165 mg of cells were incubated for 2.5 hr in Warburg flasks containing 4% defatted Pentex albumin and 2.8 mM glucose. Respiration was measured over the last 2 hr. Glucose 6-phosphate and ATP were determined on perchloric acid filtrates. The values are the means of three paired experiments in the absence (□) and presence (●) of 5 μg/ml of vitamin K_5.

of adenyl cyclase, I turned to studies on oxidative metabolism of white fat cells. Naphthoquinones can serve as electron acceptors for reduced pyridine nucleotides and can bypass one or more of the sites of electron transport to donate electrons back into the electron transport chain at cytochrome *b* or *c* (2). The studies in Fig. 5 indicate that the addition of vitamin K_5 to white fat cells incubated in Pentex albumin markedly increased respiration. However, there was no decrease in the ATP content of white fat cells, even after 2.5 hr of incubation with 5 μg/ml of vitamin K_5, and the glucose 6-phosphate content was actually increased (Fig. 5).

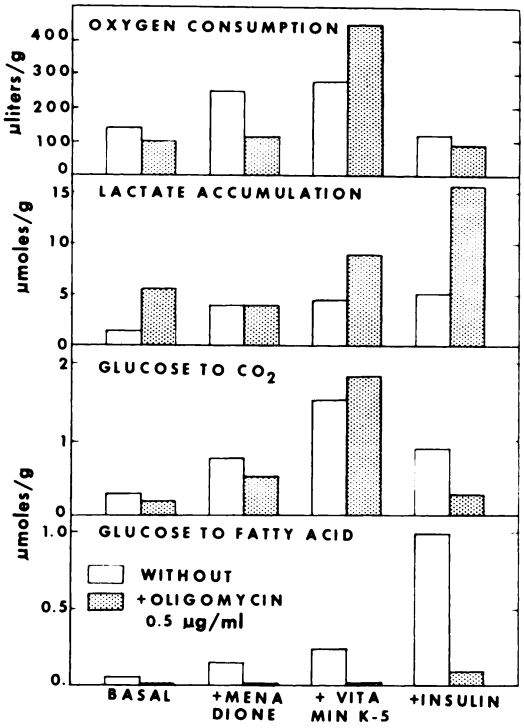

FIG. 6. *Effect of oligomycin on respiration and glucose metabolism by white fat cells incubated in the presence of menadione, vitamin K_5, or insulin*

White fat cells (145 mg/flask) isolated by digestion with 0.8 mg/ml of collagenase were incubated in Warburg flasks containing 3 ml of buffer plus 4% Armour albumin and 2.8 mM uniformly labeled glucose-^{14}C. All values are the means of two paired experiments and were obtained during a 2.5-hr incubation period except for oxygen consumption, which was measured over the last 2 hr of the incubation period. Menadione (5 μg/ml), vitamin K_5 (5 μg/ml), insulin (0.17 milliunit/ml), and oligomycin (0.5 μg/ml) were added at the start of the incubation period. Values obtained in the presence of oligomycin are shown by stippled bars, and those in its absence by unshaded bars.

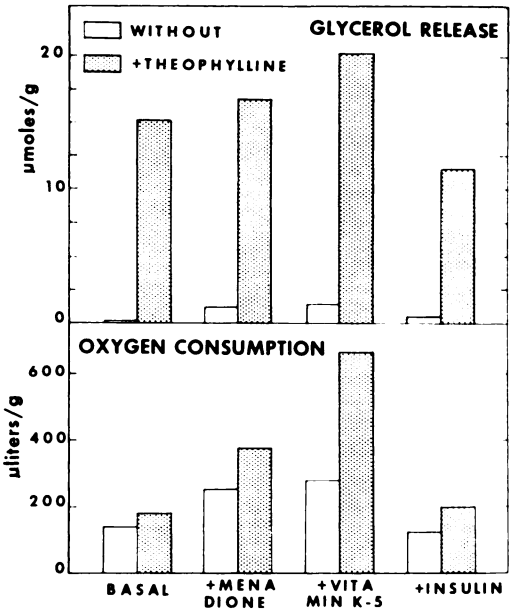

FIG. 7. *Effect of theophylline on lipolysis and glycerol release in white fat cells*

The values are taken from the same experiments described in Fig. 6. The data were obtained in the absence (unshaded bars) or presence (stippled bars) of 1 mM theophylline.

Both vitamin K_5 and menadione doubled respiration in white fat cells during a 2.5-hr incubation in Armour albumin (Fig. 6). Glucose oxidation to carbon dioxide and conversion to fatty acids and lactate accumulation were also increased. The effects of menadione and vitamin K_5 could be distinguished from that of insulin, since insulin had a greater effect on fatty acid formation, did not alter respiration, and markedly enhanced lactate accumulation in the presence of oligomycin. In the presence of oligomycin basal respiration was only slightly inhibited whereas the increase due to menadione was abolished and the effect of vitamin K_5 was potentiated. Oligomycin also affected the increase in glucose oxidation and lactate accumulation in a manner similar to vitamin K_5 and menadione. Although oligomycin completely abolished the stimulatory

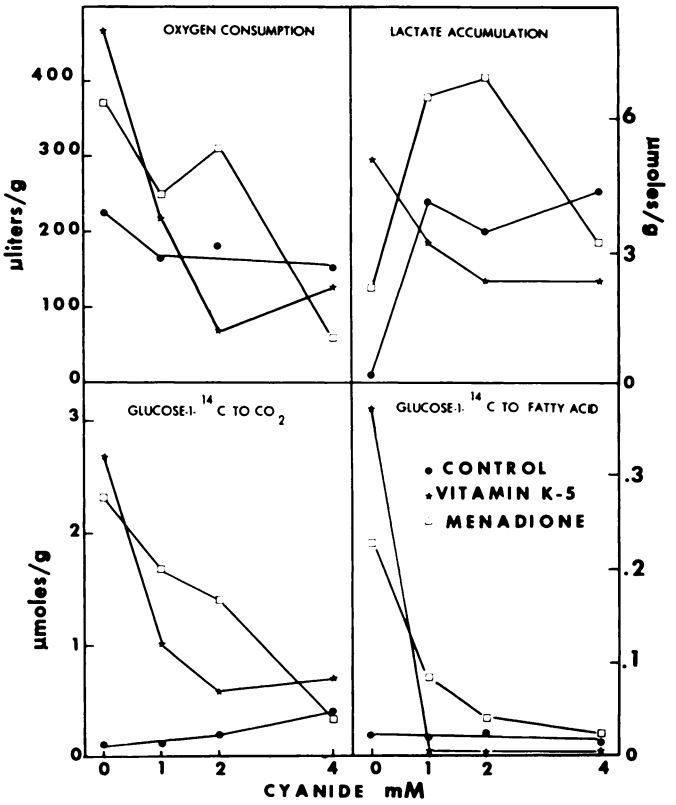

FIG. 8. *Effect of cyanide on action of menadione and vitamin* K_5

White fat cells (140 mg/flask) isolated by digestion with 0.8 mg/ml of collagenase were incubated in Warburg flasks containing 3 ml of buffer plus 4% Armour albumin and 2.8 mM glucose-1-^{14}C. The values are the means of two paired experiments except for glucose conversion to carbon dioxide and fatty acid, which are taken from a single experiment. Menadione (5 μg/ml), vitamin K_5 (5 μg/ml), and sodium cyanide were added at the start of the incubation period. The values are for a 2.5-hr incubation period except for respiration, which was measured over the last 2 hr. ○, control values; □, menadione; ★, cells incubated with vitamin K_5.

effect of insulin on fatty acid synthesis and glucose oxidation, it enhanced the effect of insulin on lactate formation.

In the same experiments as shown in Fig. 6, the effect of theophylline on respiration and lipolysis was examined in the absence and presence of menadione, vitamin K_5, and insulin. The activation of respiration by both menadione and vitamin K_5, especially the latter, was even greater in the presence of theophylline, despite the finding that neither agent inhibited the lipolytic action of theophylline in these experiments (Fig. 7). Insulin had no effect except to inhibit the lipolytic action of theophylline.

Possibly the increased respiration produced by menadione and vitamin K_5 could be attributed to interaction with enzymes such as the hepatic microsomal NADPH oxidase. Menadione can be reduced by NADPH in the presence of this enzyme, with nonenzymatic oxidation of the semiquinone back to the quinone accompanied by liberation of hydrogen peroxide (16). If this occurs in fat cells, the action of menadione might be cyanide-insensitive. However, the stimulation of glucose oxidation, fatty acid biosynthesis, and respiration by either menadione or vitamin K_5 was cyanide-sensitive (Fig. 8). Low concentrations of cyanide had a greater effect on the increase due to vitamin K_5 than on menadione

action (Fig. 8). Cyanide had little inhibitory action on basal oxygen consumption, and enhanced both basal glucose-1-^{14}C oxidation and lactate accumulation.

If naphthoquinones donate electrons into the respiratory chain at the level of cytochrome *c*, the increased respiration due to these compounds should be relatively insensitive to antimycin A, which inhibits electron transport in the cytochrome *b* to *c* region (17). Antimycin A (3 μg/ml) blocked basal respiration by about 50% and preferentially inhibited the increased oxygen consumption due to menadione. Antimycin A reduced by 75% the increase in glucose-1-^{14}C oxidation without affecting basal or vitamin K_5-stimulated glucose oxidation. Fatty acid synthesis was completely inhibited by antimycin A (Table 4).

Because of the finding that the addition of menadione *in vitro* depletes erythrocyte glutathione, the effect of adding exogenous reduced glutathione to fat cells at concentrations similar to those present in plasma was examined. The results (Table 5) indicate that glutathione, like other thiols (18),

TABLE 4

Preferential inhibition of menadione action by antimycin A

White fat cells (200 mg/flask) isolated by digestion with 0.8 mg/ml of collagenase in buffer containing 4% Armour albumin and 2.8 mM glucose were incubated for 2.5 hr in the same buffer containing glucose-1-^{14}C. All agents were added at the start of the experiment, and respiration was measured over the last 2 hr.

Measurement	Antimycin A	O_2 consumption	Glucose-1-^{14}C conversion to CO_2	Glucose-1-^{14}C conversion to fatty acid
	μg/ml	*μl/g/2 hr*	*μmoles/g*	*μmole/g*
Basal	0	128	0.35	0.03
	3	62	0.44	<0.001
Change due to vitamin K_5 (5 μg/ml)	0	+277	+1.84	+0.15
	3	+166	+1.96	0
Change due to menadione (5 μg/ml)	0	+208	+1.62	+0.18
	3	+49	+0.43	0

TABLE 5

Effect of reduced glutathione on action of vitamin K_5 and menadione

White fat cells (130 mg/flask) isolated by digestion with 0.8 mg/ml of collagenase were incubated in Warburg flasks containing 3 ml of buffer plus 4% Armour albumin and 2.8 mM glucose-1-^{14}C.

Addition	Measurement	Glucose oxidation to CO_2	Glucose conversion to fatty acid	Glucose conversion to glyceride-glycerol	Lactate accumulation	O_2 consumption
		μmoles/g	*μmole/g*	*μmoles/g*	*μmoles/g*	*μl/g*
None	Basal	0.11	0.03	0.40	0.30	197
	Change due to vitamin K_5 (5 μg/ml)	+2.56	+0.34	+0.45	+4.12	+331
	Change due to menadione (5 μg/ml)	+2.19	+0.20	+0.06	+2.22	+230
Glutathione (0.3 mg/ml)	Basal	0.69	0.30	1.35	2.21	212
	Change due to vitamin K_5 (5 μg/ml)	+1.45	0	−0.11	+2.69	+182
	Change due to menadione (5 μg/ml)	+1.20	0	−0.87	+2.99	+204

TABLE 6

Effect of menadione and vitamin K_5 on pyruvate metabolism

White fat cells (50 mg/flask) were isolated by digestion with 0.5 mg/ml of collagenase and then incubated for 1 hr in 1.5 ml of buffer containing 4% albumin plus 2 mM pyruvate, 2.8 mM glucose, or both. The basal values are the means of four paired experiments, and the changes due to menadione or vitamin K_5 are the means ± standard errors of the paired differences.

Measurement	Labeled substrate	Unlabeled substrate	Glucose conversion to CO_2	Pyruvate-1-^{14}C conversion to CO_2	Lactate accumulation
			μmoles/g	*μmoles/g*	*μmoles/g*
Basal	Glucose-U-^{14}C[a]	None	0.37		0.25
	Glucose-U-^{14}C	Pyruvate	0.06		
	Pyruvate-1-^{14}C	None		7.62	1.97
	Pyruvate-1-^{14}C	Glucose		9.60	
	Glucose-U-^{14}C + pyruvate-1-^{14}C	None			1.94
Change due to menadione (5 μg/ml)	Glucose-U-^{14}C	None	+0.77 ± 0.20		+0.89 ± 0.20
	Glucose-U-^{14}C	Pyruvate	+0.38 ± 0.12		
	Pyruvate-1-^{14}C	None		+0.2 ± 1.0	−0.68 ± 0.20
	Pyruvate-1-^{14}C	Glucose		+1.22 ± 0.50	
	Glucose-U-^{14}C + pyruvate-1-^{14}C	None			+0.07 ± 0.30
Change due to vitamin K_5 (5 μg/ml)	Glucose-U-^{14}C	None	+1.22 ± 0.40		+1.26 ± 0.35
	Glucose-U-^{14}C	Pyruvate	+0.73 ± 0.30		
	Pyruvate-1-^{14}C	None		+0.4 ± 0.70	−1.14 ± 0.40
	Pyruvate-1-^{14}C	Glucose		+1.57 ± 0.80	
	Glucose-U-^{14}C + pyruvate-1-^{14}C	None			+0.01 ± 0.10

[a] Glucose-U-^{14}C denotes the uniformly labeled compound.

is able to mimic the action of insulin on fat cells incubated in the presence of buffer containing albumin. However, glutathione did not modify the stimulatory effect of vitamin K_5 or menadione on respiration, glucose oxidation, or lactate formation.

The effects of menadione and vitamin K_5 on lactate accumulation and oxidative metabolism by fat cells in the presence of pyruvate were examined (Table 6). Neither menadione nor vitamin K_5 affected the decarboxylation of pyruvate-1-^{14}C as measured by carbon dioxide formation. Both agents decreased lactate formation in the presence of 2 mM pyruvate as the sole substrate, and increased lactate formation in the presence of 2.8 mM glucose (Table 6). In the presence of both substrates menadione and vitamin K_5 had little effect on lactate formation, and the amount of uniformly labeled glucose converted to carbon dioxide was decreased, which probably represents dilution of the acetyl-CoA pool by unlabeled acetyl-CoA derived from pyruvate decarboxylation. The reduction in lactate formation by menadione and vitamin K_5 in the presence of pyruvate may reflect decreased levels of reduced pyridine nucleotides in the cytoplasm.

DISCUSSION

The present results confirm the findings that vitamin K_5 was a potent stimulator of the accumulation of both labeled (5) and total (19) cyclic AMP in white fat cells in the presence of catecholamines and theophylline. They indicate that it is not vitamin K_5 but products of its oxidation, such as menadione, which are responsible for the accumulation of cyclic AMP. Because of the ease with which vitamin K_5 undergoes autoxidation in air, it is unlikely that this would have been noted except for the finding that Armour albumin contains

an antioxidant. Kuo (5) did observe stimulation of cyclic AMP accumulation due to vitamin K_5 in buffer containing Armour albumin, but he dialyzed the albumin extensively prior to use. The hypothesis that an antioxidant was removed during dialysis was confirmed by studies in which it was found that oxidation of vitamin K_5 readily occurred in buffer containing dialyzed Armour albumin.[1]

The ability of menadione to increase cyclic AMP accumulation is apparently not due to a direct stimulation of adenyl cyclase activity. In contrast, fluoride stimulates the adenyl cyclase activity of fat cell ghosts (12) but inhibits the accumulation of cyclic AMP by intact fat cells (Table 1). Only in one series of experiments (20) has a stimulatory effect of fluoride on cyclic AMP accumulation been seen in white fat cells, and that was in cells which had been incubated for 3 hr and were apparently damaged. Subsequent experiments[1] have failed to yield any effect of fluoride in intact fat cells (Table 1). Menadione thus affects cyclic AMP accumulation in intact cells while fluoride affects that of damaged cells or "ghosts."

Naphthoquinones have been shown to be uncouplers of oxidative phosphorylation in isolated rat liver mitochondria (21). The question arises whether other uncouplers of oxidative phosphorylation, such as valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone, might mimic the action of menadione on cyclic AMP. However, we have been unable to detect any stimulation of cyclic AMP accumulation due to carbonyl cyanide *m*-chlorophenylhydrazone,[1] while valinomycin has been shown to inhibit cyclic AMP accumulation in white fat cells (22, 23).

Menadione may preferentially compete with flavoproteins for the electrons from NADH or NADPH and then donate the electrons to cytochrome *b* or *c* or be oxidized by molecular oxygen back to the quinone, with liberation of hydrogen peroxide (16, 17). The sensitivity of menadione action to antimycin A and cyanide suggests that it donates electrons at the level of cytochrome *b*.

The major difference between the effects of menadione and vitamin K_5 on oxidative metabolism was the ability of oligomycin to block the stimulation of respiration due to menadione while potentiating that produced by vitamin K_5. If oligomycin acts on white fat cells primarily by blocking the formation of ATP from the conserved energy of electron transport, it is possible that menadione-stimulated respiration involves transfer of electrons into energy-conserving sites in the cytochrome pathways whereas vitamin K_5-induced respiration is completely uncoupled from oxidative phosphorylation. These conclusions are supported by the preferential inhibition by antimycin A of menadione action.

Menadione stimulates respiration via processes sensitive to oligomycin and antimycin A while increasing the maximal rate of cyclic AMP accumulation in the presence of catecholamines and theophylline. In contrast, vitamin K_5 decreases cyclic AMP accumulation, and its effects on respiration are largely insensitive to oligomycin and antimycin A. However, the two compounds share the ability to stimulate glucose oxidation. Whether the delayed antilipolytic action of vitamin K_5 is secondary to stimulation of glucose metabolism is not known, but it is apparently unrelated to effects on cyclic AMP accumulation.

Menadione at concentrations comparable to those used in the present experiments (30 μM) was originally shown to increase the oxidation of glucose by the pentose cycle and decrease aerobic glycolysis of brain slices (24, 25). Kuo and associates (4, 5) found that deoxyfrenolicin, a novel 1,4-naphthoquinone derivative with an epoxide structure, stimulated glucose oxidation and cyclic AMP accumulation. The effects of menadione reported here are similar to those of deoxyfrenolicin, which is not surprising since both are 1,4-naphthoquinone derivatives.

Kuo, Dill, and Holmlund (4) found that while naphthoquinones such as vitamin K_5 and deoxyfrenolicin mimicked the action of insulin on glucose oxidation, their mecha-

[1] Unpublished observations.

nisms of action were different from that of insulin. Naphthoquinones had a less stimulatory effect on fatty acid synthesis than did insulin, and stimulated glycogenolysis while insulin was inhibitory (4). The results in Tables 2 and 5 and Fig. 6 confirm the finding that naphthoquinones had a smaller effect on fatty acid synthesis than did insulin. Furthermore, under conditions in which insulin and naphthoquinones had similar effects on glucose oxidation, insulin was able to stimulate leucine incorporation into fat cell protein by over 100% while menadione actually inhibited protein synthesis.[1] These findings all support the hypothesis that the mechanisms by which naphthoquinones affect the metabolism of fat cells have little similarity to those involved in insulin action.

The increased rate of glucose oxidation and glycolysis suggests that the rate of glucose transport was increased, since it is the rate-limiting factor in glucose metabolism in the basal state. Perhaps the increased utilization of cytoplasmic reducing equivalents by fat cell mitochondria results in an alteration in the rates of glucose transport and cyclic AMP accumulation.

The present results suggest a regulatory role for oxidation-reduction carriers in the intracellular regulation of glucose metabolism, cyclic AMP accumulation, and lipolysis in fat cells. Possibly natural oxidation-reduction carriers such as quinones or flavin derivatives play a more important role in membrane transport and mitochondrial-cytoplasmic interactions than has been realized. In intact fat cells the availability of energy for adenyl cyclase and glucose transport may be very important, and menadione could disturb the energy balance of the cell by affecting reduced pyridine nucleotide utilization by mitochondria. The addition of menadione might result in an increased transport of reducing equivalents between mitochondria and the plasma membrane through the cytoplasm by substituting for the natural oxidation-reduction carriers.

Kuo (5) suggested that the effects of deoxyfrenolicin and vitamin K_5 could be due to interference with oxidative phosphorylation. Since none of the effects of naphthoquinones were accompanied by a depletion of total ATP, it is clear that the situation is rather complex. One could invoke compartmentation of ATP to explain the findings. However, it appears more likely, as suggested by Schmidt and Katz (26), that for intact adipose tissue the transport of hydrogen or reducing equivalents between compartments is an important factor in the control of glucose transport, lipolysis, and cyclic AMP accumulation in fat cells. This may be a more important control site in white fat cells than in other cells because of the reported absence of shuttles to transfer hydrogen equivalents into mitochondria from the cytoplasm (26). The available data are compatible with the hypothesis that the effects of naphthoquinones may be the result of their ability to act as shuttles for transfer of reducing equivalents between the cytoplasm and the other compartments within fat cells.

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