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INHIBITION OF OVARIAN CHOLESTEROL ESTERIFICATION BY DIETHYLAMINOETHYL DIPHENYLVALERATE (SKF 525-A) AND OTHER IONIC AMPHIPATHIC AMINES

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Abstract—The effects of diethylaminoethyl diphenylvalerate HCl (SKF 525-A), 2,2‴-[(1-methyl-4,4-diphenylbutylidene)bis(*p*-phenyleneoxy)] bistriethylamine oxalate (SQ 10,591), carnitine and palmitoyl carnitine on cholesterol esterification were studied in the mitochondrial and microsomal fractions from both interstitial tissue and corpora lutea of 8-day-pregnancy rabbit ovaries. In the mitochondria at pH 4·4 and without added cofactors, SKF 525-A and SQ 10,591 at concentrations of 10^{-3} to 5×10^{-4} M significantly inhibited esterification of cholesterol and palmitic acid substrates; palmitoyl carnitine inhibited the reaction at concentrations of 10^{-3} M to less than 10^{-5} M, whereas carnitine had no effect. In the microsomal fractions at pH 7·1, a palmitoyl CoA substrate was incorporated into cholesteryl esters at a more rapid rate than was a palmitic acid substrate with added ATP and CoA cofactors. Incorporation of the cholesterol, palmitic and palmitoyl CoA substrates into microsomal cholesteryl esters was inhibited by SKF 525-A and SQ 10,591 at concentrations greater than 5×10^{-4} M, but not inhibited at all by palmitoyl carnitine or carnitine.

ESTERIFICATION of cholesterol in the early pregnancy rabbit ovary occurs by two enzymatic reactions. One is an ATP and CoA-dependent acyltransferase, localized primarily in the microsomal compartment, and the other is a non-cofactor requiring enzyme active at low pH and is predominantly in the mitochondrial fraction.[1] The rate of esterification by both these enzymatic mechanisms is increased during early pregnancy[2] and may be the mechanism whereby the content of ovarian cholesteryl esters is increased during this period. It has been theorized that these cholesteryl esters provide a storage form of cholesterol[3] and may assure the provision of increased quantities of substrate for steroid hormone synthesis during pregnancy. Pharmacologic inhibition of cholesteryl ester synthesis could provide data to further elucidate the role of this process in early pregnancy.

Diethylaminoethyl diphenylvalerate HCl (SKF 525-A) (Fig. 1) has been shown to inhibit a number of microsomal drug-metabolizing enzymes.[4] In addition, this compound is an inhibitor of cholesterol biosynthesis[5,6] (at the stage of squalene conversion to lanosterol) and also inhibits oxidation of intermediates in the side chain degradation reaction pathways in mitochondria.[7] The oxidation of long chain fatty acids by mitochondria, however, is stimulated by SKF 525-A, an effect similar to that of DL-carnitine;[7] at a low concentration of carnitine, SKF 525-A seems to antagonize the latter's effect on fatty acid oxidation.

SQ 10,591 2,2″-[(1-methyl-4,4-diphenylbutylidene)bis(*p*-phenyleneoxy)]bistriethylamine oxalate is another amphipathic amine compound which has been shown to also inhibit cholesterol biosynthesis by blocking the conversion of mevalonate to lanosterol.[8] When administered to rats, SQ 10,591 resulted in greater decreases in plasma and liver cholesteryl esters than in free cholesterol, suggesting an inhibition of cholesterol esterification.

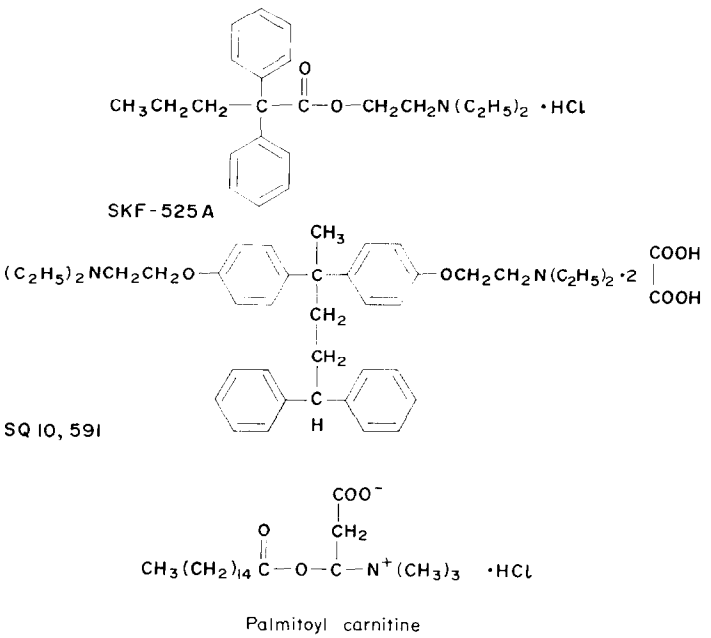

FIG. 1. Structural formulas of SKF 525-A, SQ 10,591 and palmitoyl carnitine.

The aims of the present study were to determine the effects *in vitro* of these compounds on the ovarian mitochondrial and microsomal cholesterol-esterifying enzyme systems.

MATERIALS AND METHODS

Female New Zealand white rabbits, 12–18 months of age were mated, and the ovaries removed on day 8 of pregnancy. Only ovaries having evident corpora lutea of pregnancy were used for the assay experiments.

The corpora lutea were dissected free of the interstitial tissue, and each of these portions was homogenized separately with a ground glass mortar and pestle in 0·25 M sucrose + 0·001 M EDTA at a concentration of 100 mg/ml. The homogenates were centrifuged for 10 min at 500 *g*, the supernatant was then centrifuged at 12,000 *g* for 15 min and then recentrifuged at 18,000 *g* for 15 min. The floating lipid granule layer and the supernatant were withdrawn separately with a capillary pipet. The 12,000 *g* mitochondrial precipitate was resuspended in an equal volume of 0·25 M sucrose, and recentrifuged. The 12,000 *g* supernatant was centrifuged at 104,000 *g* for 60 min. The floating lipid layer was carefully removed with a capillary pipet, and then the remainder of the supernatant (the cytosol fraction) removed separately. The

microsomal precipitate was resuspended in 0·15 M KCl and recentrifuged. The washing (supernatant) from this centrifugation was discarded.

For assay of esterification by the acyltransferase reaction, 0·5-ml aliquots of the subcellular fractions were mixed with 0·5-ml aliquots of 0·2 M phosphate buffer, pH 7·1, 12 μmoles ATP, 0·6 μmole CoA and 10 μmoles $MgCl_2$. SKF 525-A, DL-carnitine, DL-palmitoyl carnitine HCl (Calbiochem, A grade) or SQ 10,591 were added to the incubation solution over a concentration range of 10^{-6} to 10^{-3} M. The palmitoyl carnitine was dissolved in 10 μl propylene glycol before addition to the incubations. Control experiments for this series were done by adding 10 μl propylene glycol without the palmitoyl carnitine. A cholesterol-4-^{14}C substrate (0·05μCi, 10 nmoles), palmitic-1-^{14}C acid substrate (0·05 μCi, 10 nmoles) or oleic-1-^{14}C acid substrate (0·05μCi, 10 nmoles) in 10 μl acetone was added to each and the tubes were incubated at 37 for 2 hr. In other incubations, a palmitoyl-1-^{14}C coenzyme A substrate (0·05 μCi, 10 nmoles) was added to the phosphate buffer. All substrates were purchased from the New England Nuclear Co. In some experiments $^{14}CO_2$ was collected in a center well containing 20% NaOH. Control aliquots were heated at 90 for 10 min prior to incubation. The incubations were stopped by addition of 10 ml of chloroform–methanol (2:1) and the lipids extracted by homogenization with a ground glass mortar and pestle, centrifuged, and the residue was extracted again with 10 ml chloroform methanol. After preliminary purification of the samples by the procedure of Folch *et al.*,[9] they were evaporated to dryness under an N_2 stream, dissolved in chloroform and the lipid fractions separated by thin-layer chromatography on microscope slides coated with Silica gel H (Brinkmann) using hexane–ethyl ether–acetic acid (80:20:1) as the developing solvent. The free and esterified cholesterol zones were scraped into vials and their radioactivities determined by liquid scintillation counting in a PPO–POPOP–toluene* scintillation solution using a Packard 3314 automatic refrigerated liquid scintillation spectrometer. Quenching was monitored by subsequent addition of internal standards.

For assay of the non-cofactor esterification reaction, 0·5-ml aliquots of the ovarian subcellular fractions were incubated with 0·5 ml of 0·2 M citrate-phosphate buffer, pH 4·4, containing 0·05 μCi, 10 nmoles of the cholesterol-4-^{14}C, palmitic-^{14}C or oleic-1-^{14}C substrate for 2 hr at 37 . Samples were then processed as in the assay of the preceding acyltransferase enzyme.

For assay of cholesteryl ester hydrolase, 0·5-ml aliquots of the fractions were incubated in 0·5 ml of 0·1 M Tris-maleate buffer at pH 6·6 with 3 mg fat-free albumin and 10 μl of cholesteryl-4-^{14}C-oleate in acetone (New England Nuclear, 0·05 μCi, 10 nmoles in each tube), for 2 hr at 37 . Control aliquots were heated at 90 for 10 min prior to incubation. The incubations were stopped by addition of 10 ml of ethanol–acetone (1:1). Free cholesterol in aliquots of these extracts was precipitated as the digitonide complex, centrifuged, washed with ether, dissolved in methanol and the radioactivities were determined as above in both this free cholesterol and in a total cholesterol aliquot.

Free and esterified cholesterol contents of the fractions were determined fluorometrically by the method of Zeitman[10] after separation of these compounds by thin-layer chromatography as described above. Free fatty acids were determined colori-

* PPO, 2·5-diphenyloxazole; POPOP, 1,4-bis-[2·5-phenyloxazolyl]benzene.

metrically as described by Mahadevan *et al.*[11] Protein content of each fraction was analyzed by the method of Lowry *et al.*[12] adapted for the autoanalyzer.

The probabilities (P) that apparent differences in the data were due to chance were calculated by the *t*-test.

RESULTS

In Table 1 are indicated the degrees of incorporation of ^{14}C-labeled cholesterol, palmitic acid, oleic acid or palmitoyl coenzyme A into cholesteryl esters by mitochondria and microsomes of both interstitial tissue and corpora lutea of the eight-day-pregnancy rabbit ovaries. The mitochondrial reaction was assayed without

TABLE 1. ESTERIFICATION OF CHOLESTEROL BY OVARIAN SUBCELLULAR FRACTIONS*

Substrate	Cholesterol-4-^{14}C	Palmitic-1-^{14}C	Oleic-1-^{14}C	Palmitoyl-1-^{14}C CoA
Mitochondria				
Interstitial	1·31 ± 0·12	0·82 ± 0·19	0·75 ± 0·09	0·05 ± 0·01
Corpora lutea	1·16 ± 0·14	0·72 ± 0·11	0·71 ± 0·15	0·04 ± 0·01
Microsomes				
Interstitial	0·94 ± 0·11	0·64 ± 0·07	0·64 ± 0·08	6·23 ± 0·75
Corpora lutea	0·87 ± 0·14	0·51 ± 0·08	0·49 ± 0·06	5·97 ± 0·52

* Mitochondrial incubations were done at pH 4·4 for 2 hr at 37° in 1·0 ml of 0·1M citrate-phosphate buffer with no added cofactors. Microsomal incubations were done at pH 7·1 for 2 hr at 37° in 1·0 ml of medium containing 0·1 M phosphate buffer, 6 μmoles ATP, 0·3 μmole CoA, and 5 μmoles $MgCl_2$. Incubations with palmitoyl-^{14}C CoA were done without added cofactors. Each substrate added contained 0·05 μCi and 10 nmoles. Figures given are mean nmoles substrate esterified·mg protein·hr ± standard deviations of incubations from six separate specimens; nmoles esterified are calculated assuming no equilibrium with endogenous substrate.

added cofactors and at pH 4·4, which was the optimal pH for this reaction. The extent of esterification with cholesterol was greater than with either palmitic or oleic acids; these two fatty acids showed comparable degrees of incorporation. Esterification with palmitoyl coenzyme A was very low in the mitochondria at this pH. The reaction was assayed at 30-min intervals up to 120 min and was found to be linear with each substrate during this time period.

Esterification by the microsomal fractions was assayed with ATP and coenzyme A added, and at pH 7·1, which was the optimal pH for this reaction. The reaction with the palmitoyl-^{14}C coenzyme A substrate was assayed at pH 7·1 without any added cofactors. Esterification was most rapid with the latter substrate, and was lowest with the palmitic and oleic acid substrates. The reaction rate was linear over a 120-min period with each substrate. Although rates of esterification of all substrates by both mitochondria and microsomes were generally slightly higher in the interstitial tissue as compared to the corpora lutea, these differences were only of borderline significance.

Freezing the microsomal fractions at −15° overnight resulted in 80–90 per cent inactivation of esterifying activity at pH 7·1 with each of the substrates. The esterification by the mitochondrial fractions at pH 4·4 with no added cofactors was not affected by freezing.

Addition of 10–30 nmoles of unlabeled palmitic or oleic acids with the 10 nmoles cholesteryl-^{14}C substrate or of 10–30 nmoles unlabeled cholesterol with the palmitic-

^{14}C, oleic-^{14}C or palmitoyl-^{14}C CoA substrates had no significant effect on the reactions in either the mitochondria or microsomes.

In Table 2 are indicated the effects of 10^{-4} M concentrations of SKF 525-A, DL-carnitine, DL-palmitoyl carnitine and SQ 10,591 on mitochondrial esterification

TABLE 2. EFFECTS OF ADDED COMPOUNDS ON OVARIAN MITOCHONDRIAL ESTERIFICATION OF CHOLESTEROL AND PALMITIC ACID SUBSTRATES*

Substrate	Cholesterol-4-^{14}C	Palmitic-1-^{14}C
Interstitial +		
No additions	1·32 ± 0·14	0·81 ± 0·13
SKF 525-A	0·70 ± 0·09	0·48 ± 0·06
DL-Carnitine	1·35 ± 0·13	0·83 ± 0·09
Palmitoyl carnitine	0·22 ± 0·08	0·11 ± 0·01
SQ 10,591	0·68 ± 0·15	0·39 ± 0·05
Corpora lutea +		
No additions	1·12 ± 0·12	0·77 ± 0·13
SKF 525-A	0·64 ± 0·17	0·58 ± 0·10
DL-Carnitine	1·17 ± 0·21	0·71 ± 0·14
Palmitoyl carnitine	0·15 ± 0·06	0·23 ± 0·04
SQ 10,591	0·55 ± 0·08	0·40 ± 0·05

* Esterification was assayed at pH 4·4 as described in Table 1. Each compound was added to the media at a concentration of 10^{-4} M.

of cholesterol and palmitic acid substrates at pH 4·4 with no added cofactors. Palmitoyl carnitine had the greatest inhibitory effect (83–86 per cent inhibition with both the cholesterol and palmitic acid substrates). Carnitine produced no inhibition of esterification, whereas SKF 525-A and SQ 10,591 at 10^{-4} M partially inhibited the esterification reaction. The degrees of inhibition were similar in mitochondria of both the interstitial tissue and the corpora lutea.

The effects of concentration of each compound on the degree of inhibition of incorporation of cholesterol-^{14}C into the interstitial mitochondrial cholesteryl esters are indicated in Fig. 2. Palmitoyl carnitine showed the most potent inhibitory effects.

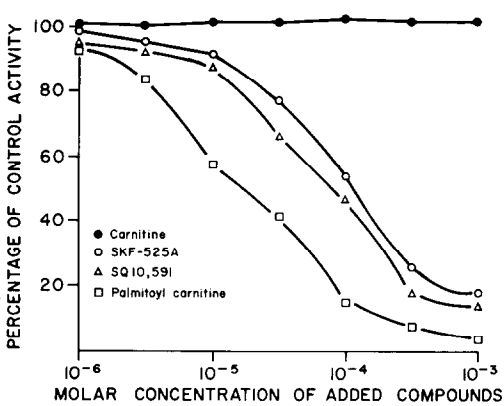

FIG. 2. Esterification of a cholesterol-^{14}C substrate by ovarian interstitial mitochondria at pH 4·4 with no added cofactors. Incubation conditions are given in Table 1. Activities are expressed as percentages of the control esterification.

TABLE 3. EFFECTS OF ADDED COMPOUNDS ON ESTERIFICATION OF CHOLESTEROL BY OVARIAN MICROSOMES*

Substrate	Cholesterol-4-^{14}C	Palmitic-1-^{14}C	Palmitoyl-1-^{14}C CoA
Interstitial +			
No additions	0·94 ± 0·14	0·65 ± 0·07	6·21 ± 0·65
SKF 525-A	0·61 ± 0·10	0·37 ± 0·04	4·15 ± 0·60
DL-Carnitine	0·97 ± 0·18	0·61 ± 0·12	6·36 ± 0·81
Palmitoyl carnitine	1·10 ± 0·11	0·65 ± 0·10	6·80 ± 0·90
SQ 10,591	0·56 ± 0·06	0·41 ± 0·08	3·79 ± 0·48
Corpora lutea +			
No additions	0·87 ± 0·13	0·52 ± 0·09	5·92 ± 0·74
SKF 525-A	0·52 ± 0·09	0·29 ± 0·05	3·60 ± 0·47
DL-Carnitine	0·84 ± 0·20	0·54 ± 0·12	5·80 ± 0·26
Palmitoyl carnitine	0·94 ± 0·13	0·57 ± 0·07	6·17 ± 0·53
SQ 10,591	0·58 ± 0·14	0·27 ± 0·04	3·30 ± 0·28

* Esterification was assayed at pH 7·1 as described in Table 1. Each compound was added to the media at a concentration of 10^{-4} M.

whereas carnitine in the range of 10^{-6} to 10^{-3} M had no significant effects on esterification. SKF 525-A and SQ 10,591 showed parallel relationships of concentration to inhibition of the reaction. Significant inhibition appeared to begin at 5×10^{-4} M, and at 10^{-3} M esterification was less than 20 per cent of the control values.

The effects of addition of these compounds on esterification of cholesterol-^{14}C, palmitic-^{14}C and palmitoyl-^{14}C CoA by the microsomal fractions at pH 7·1 are indicated in Table 3. ATP and CoA were added to incubations with the cholesterol and palmitic acid substrates but not with the palmitoyl CoA. SKF 525-A and SQ 10,591 at concentrations of 10^{-4} M resulted in significant inhibition of incorporation of each substrate into both interstitial and corpora lutea cholesteryl esters. Unlike the effects on mitochondria at pH 4·4, palmitoyl carnitine had no inhibitory effect on incorporation of the cholesterol or palmitic acid precursors into cholesteryl esters by the microsomal fractions; palmitoyl CoA incorporation was also not significantly affected.

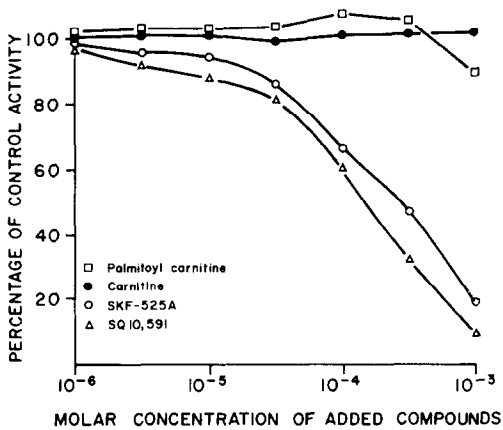

FIG. 3. Esterification of the palmitoyl-^{14}C CoA substrate by ovarian interstitial microsomes at pH 7·1 with no added cofactors. Incubation conditions are given in Table 1. Activities are expressed as percentages of the control esterification.

In Fig. 3 are shown the effects of 10^{-6} to 10^{-3} M concentrations of SKF 525-A, SQ 10,591, palmitoyl carnitine and carnitine on incorporation of the palmitoyl-^{14}C CoA substrate into the interstitial microsomal fraction. Significant inhibition by SKF 525-A and SQ 10,591 seemed to begin at 10^{-4} M, and esterification at 10^{-3} M was less than 20 per cent of the control. Carnitine had no effects at any of the concentration levels, and palmitoyl carnitine induced a slight rise (of borderline significance) in activity at 10^{-4} M.

No significant effects on cholesteryl ester hydrolase activities of either the mitochondria or microsomes were induced by any of the added compounds, and no differences in oxidation of any of the fatty acid substrates to $^{14}CO_2$ were noted.

DISCUSSION

As found in previous investigations,[1] the low pH, non-cofactor esterification reaction was highest in the mitochondria, and the ATP–CoA-dependent acyltransferase reaction was highest in the microsomal fraction; therefore, most assays with the added compounds were done utilizing the mitochondria for the former reaction and the microsomes for the latter. Esterification of the palmitoyl CoA substrate by the microsomal fraction was found to occur at a much faster rate than esterification with the palmitic acid substrate with added ATP and CoA cofactors, suggesting that the formation of acyl CoA thioesters is a rate-limiting step in this reaction sequence. The inactivation of microsomal esterification by freezing may have resulted from denaturation of the acyl CoA synthetase or the acyltransferase enzymes, or by alteration of the spatial arrangement of these enzymes within the microsomal membranes such as to interrupt the reaction sequence.

SKF 525-A and SQ 10,591 at concentrations of 5×10^{-4} to 10^{-3} M, and palmitoyl carnitine at 10^{-5} to 10^{-3} M each inhibited the incorporation of cholesterol and palmitic acid into mitochondrial cholesteryl esters. In contrast to palmitoyl carnitine, carnitine alone showed no inhibition at any of the concentration levels studied, suggesting that the amphipathic properties of the acylcarnitine may be essential for this effect. Similar inhibitory effects by SKF 525-A and SQ 10,591 were noted on incorporation of cholesterol, palmitic acid and palmitoyl CoA substrates into the microsomal cholesteryl esters. These effects on the mitochondria and microsomes appeared to be a true inhibition of cholesterol esterification, since the rate of hydrolysis of cholesteryl esters was not stimulated by any of the compounds. Although SKF 525-A has previously been reported to stimulate fatty acid oxidation to CO_2 by mouse liver and beef heart mitochondria,[7] this effect was not observed in the ovarian mitochondria in the present study and neither was increased $^{14}CO_2$ from the substrate fatty acids produced by the other added compounds.

The mechanisms whereby these compounds produced their inhibitory effects could be via their interaction with the substrates or enzymes involved in the esterification reaction or on the permeability of the mitochondrial or microsomal membranes to these substrates. Compounds with surface active properties have been observed both to enhance and inhibit reactions of enzymes with lipid substrates.[14] Surface tensions of aqueous solutions containing SKF 525-A have been shown to progressively decrease from a baseline of 70 dynes/cm at 10^{-6} M to 46 dynes/cm at 5×10^{-3} M (the critical micellar concentration).[13] This decrement in surface tension does occur

over the same range of concentration at which both SKF 525-A and SQ 10,591 showed their inhibitory effects in the present experiments. In other studies, SKF 525-A has been shown to interact with cholesterol monolayers at concentrations of 6×10^{-5} M, penetrating the films and changing their character from condensed to liquid expanded films.[15] This effect could conceivably have limited the diffusion of the cholesterol-^{14}C substrate to the sites of esterification in the present experiments. An alternative possibility is that the SKF 525-A, SQ 10,591 and palmitoyl carnitine may induce the formation of mixed micelles with the cholesterol or fatty acid substrates. If the enzymes involved in cholesterol esterification are of the type IV characterized by Gatt *et al.*[16] as acting on only monomeric and not on micellar forms of lipid substrates, formation of the micelles could decrease the velocity of the esterification reaction. Contrary to the idea of an inhibitor–substrate interaction being the mechanism of inhibition is the fact that incorporation of cholesterol, fatty acid and acyl CoA substrates, compounds of widely different polarities and aqueous solubilities, were inhibited to similar degrees by each of the added compounds. Also, the addition of a non-ionic surface active agent, Tween 80 (polyoxyethylene sorbitan mono-oleate) at concentrations of 0·25 μg/ml and 0·5 μg/ml produced no inhibitory effects on esterification of any of the substrates in the mitochondria or microsomes.

Direct effects on the enzymes or on the enzyme–substrate interactions are other possible mechanisms of action of these compounds. Kinetic data from a previous study suggest that the inhibition of *N*-demethylation of lipid-soluble drugs by microsomes may be a competitive type of inhibition.[17] The inhibition of esterification by the cationic amphipathic compounds in the present experiments could be the result of a competition with the acyl CoA synthetase or the acyltransferase for the negatively charged fatty acid substrates. It is possible that the inhibitory effect of palmitoyl carnitine at pH 4·4 on the mitochondrial reaction and the lack of inhibition at pH 7·1 in both the mitochondria and microsomes are in part attributable to the different charge on the palmitoyl carnitine at these differing pH values.

It has been postulated that a lipid boundary layer may limit the penetration of polar substrates into the microsomal membrane.[18] Other investigations suggest that SKF 525-A may inhibit the action of drug-metabolizing and other enzymes via an interaction with the microsomal membrane such as to alter its permeability.[19] SKF 525-A has, in fact, been shown to become strongly bound to the microsomes, such that it is not removed by dialysis or washing.[20] Addition of SKF 525-A in a membrane simulation experiment (cholesterol-lecithin monolayers on aqueous solutions) *in vitro* resulted in an interaction of the drug with the monolayer and a resulting increased surface pressure and expansion of the layer.[15] An alteration of membrane permeability would probably best explain the similar inhibitory effects of each of the compounds in the present experiment on incorporation of the diverse types of substrates that were utilized, and the similarity of effects on both mitochondria and microsomes.

Although it cannot be definitely predicted from the above studies *in vitro* whether the observed effects on ovarian cholesterol esterification of the compounds studied will be similar in intact biological systems, it has been previously observed that SQ 10,591 administered to rats resulted in not only a block in conversion of polyprenol phosphate precursors to squalene and lanosterol, but also an equally marked decrease in plasma and liver esterified cholesterol.[8] Free cholesterol concentrations in

plasma and liver were not decreased. In addition, incorporation of acetate-^{14}C into plasma and liver cholesteryl esters was decreased, but incorporation *in vivo* into free cholesterol was not decreased.[8] These data suggest that a primary effect of SQ 10,591 *in vivo* may be an inhibition of cholesterol esterification.

It has been suggested that the cholesteryl esters in the ovary may constitute a storage form of substrate for steroid synthesis,[3] which becomes available upon enzymatic hydrolysis of these esters. Compounds which inhibit the formation of cholesteryl esters may be a useful tool for elucidating the role of these cholesteryl esters in steroidogenesis, in particular during the early pregnancy period, when cholesterol-esterifying activity and cholesteryl ester levels rise markedly,[2] and there is an accompanying rise in steroidogenic activity.

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