CHOLESTEROL ESTERASE ACTIVITY OF PLEUROPNEUMONIALIKE ORGANISMS[1]

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Nutritional studies have shown that the sterol requirement of certain pleuropneumonialike organisms can be met only by a molecule similar to cholesterol in structure (Smith and Lynn, 1958). The absolute requirement of a 3-β-hydroxyl group presupposed that this group performs some necessary function. The fact that cholesteryl esters of fatty acids possess growth promoting activity indicated that pleuropneumonialike organisms might contain a cholesterol esterase. Such a cholesterol esterase could perform many functions relative to substrate permeability, detoxification of fatty acids, participation in oxidation phosphorylation by reduction and oxidation at $\Delta 5$, or could simply supply free cholesterol. Preliminary experiments indicated that pleuropneumonialike organisms possessed cholesterol esterase activity. The results presented in this report constitute an examination of the nature of this activity together with some studies designed to elucidate its possible function.

MATERIALS AND METHODS

Seven strains of pleuropneumonialike organisms representing types with different nutritional requirements were employed: strains O7, 39, 48, and Campo are human genital strains; J is a poultry strain; B-15 is a saprophytic bovine genital strain; Laidlaw B is a sewage strain. Strains B-15 and Laidlaw B, obtained from Dr. D. G. ff. Edward, Wellcome Research Laboratories, Beckenham, Kent, England, do not require any component of the lipoprotein factor for growth and are readily cultivated on extract agar or broth. The Campo strain is capable of growth when Tween 80 and starch substitute for the lipoprotein factor (Smith *et al.*, 1954). The remaining five strains have an absolute requirement for the lipoprotein factor. Strain O7 served as the representative test organism.

The cholesteryl esters employed in this study were synthesized and purified according to the

method of Swell and Treadwell (1955). The other compounds were obtained from commerical sources (Chemed, Inc., Distillation Products Industries, Nutritional Biochemicals Corporation, and California Foundation for Biochemical Research).

Resting cell suspensions were prepared as previously described (Smith, 1955). Extracts were prepared by subjecting resting cell suspensions in saline to sonic vibration in a 9 kc Raytheon magnetoconstriction oscillator for 20 min. Crude fractionation of cells into residue and supernatant fractions was accomplished by centrifugation of disrupted cells at 20,000 × G for 5 min in a Servall angle centrifuge. The supernatant fluid was decanted and reserved. The residue was washed once with saline. For experiments in which the cofactor effect of coenzyme A was tested, disrupted cells were shaken with Dowex 1-Cl and the resin removed by slow speed centrifugation so as not to sediment the insoluble cell material. Reactions were carried out in a total volume of 2.5 ml at 37 C for 6 hr in 0.06 M phosphate buffer, pH 6.5, unless otherwise indicated. Sterols and esters in amounts of 5 to 10 μmoles were added as acetone solutions. Acetone in the amount of 0.1 ml per 2.5 ml of reaction mixture was found to have no effect on the reaction. The presence of cells appeared to facilitate colloidal dispersion of the sterols. Following the reaction period, an equal volume of 1:1 acetone-methanol was added to stop the reaction. Three-tenths-ml portions were added to 9.0 ml 1:1 acetone-methanol, the denatured protein removed by centrifugation, and 3.0-ml samples used for determination of cholesterol and cholesteryl esters by the method of Wycoff and Parsons (1957). Appropriate cell and substrate controls were employed in each experiment. In experiments on inhibition of esterase activity, appropriate controls with the inhibitor exclusive of substrate were employed. In the case of esters other than cholesteryl esters, fatty acid liberation was measured by electrometric titration with standard

alkali (Goldstein *et al.*, 1948). Identical samples of the same reaction mixture were used for zero time and final titration. Since the cellular nitrogen varied somewhat between experiments, the data in most instances are presented as μmoles of free sterol or free fatty acid liberated per milligram of cellular nitrogen.

Hydroxamic acid and acyl coenzyme A formation were detected by the method of Lipmann and Tuttle (1945).

EXPERIMENTAL RESULTS

The hydrolytic activity of the seven strains employed for various fatty acid esters of cholesterol is shown in table 1. It can be noted that strains O7, 39, 48, and J, all of which require sterol for growth, possess esterase activity for all of the esters employed. The Campo strain showed relatively poor activity as did the saprophytic strains, B-15 and Laidlaw B. Growth of the saprophytic strains in the presence or absence of the lipoprotein factor did not affect their esterase activity. Maximal hydrolysis of a given ester by 2 mg cellular nitrogen normally used, resulted in the liberation of a total of 1.5 to 5.0 μmoles of free cholesterol over a period of 6 hr. That this activity is enzymatic in nature can be noted in figure 1, whereby the amount of free cholesterol liberated was dependent upon the amount of cellular nitrogen present in the reaction mixture. In addition, the incubation of cholesteryl esters with whole cells, heated for 15 min at 65 C, resulted in no liberation of free cholesterol.

Stoichiometric analysis of the reaction mixtures showed that for each mole of ester hydrolyzed, 1 mole each of free cholesterol and fatty acid is formed. Table 2 gives representative data for cholesteryl valerate; the values are corrected for endogenous cellular sterol. No loss of free cholesterol occurred. This is in accord with recovery experiments in which no loss of cholesterol was ever noted. On the other hand, total recovery of fatty acid was not obtained even at pH 6.5. Although considerable O_2 uptake is demonstrable in the presence of fatty acids after a 1- to 2-hr lag period only at alkaline pH, some fatty acid utilization must occur at pH 6.5.

Disrupted cells were found to retain essentially all the activity present in whole cells (table 3). Hydrolytic activity was apparent at all pH values tested, the degree of activity depending upon the ester employed as substrate. However, an optimal pH of 6.5 was noted for all esters (table 4).

No absolute cofactor requirement for the hydrolytic reactions could be demonstrated after dialysis of disrupted cells for 24 to 48 hr against frequent changes of distilled water. However, several surface active compounds stimulated the liberation of free cholesterol (table 5) as has been shown with the mammalian enzyme by Swell *et al.* (1953). When varying levels of lecithin or sodium cholate were employed, increased stimulation of hydrolysis occurred up to a level of 10 μmoles or to a molar ratio of sterol to cofactor of one. This stimulation of hydrolysis of cholesteryl esters was probably due to increased solubilization of the cholesteryl esters by lecithin and so-

TABLE 1

Hydrolysis of cholesteryl esters by various strains of pleuropneumonialike organisms

Strain	Cholesteryl Ester									
	Acetate	Propionate	Butyrate	Valerate	Caproate	Caprylate	Laurate	Palmitate	Stearate	Oleate
	Free cholesterol formed per mg N in 6 hr									
	μmoles									
O7	0.84	0.54	1.38	0.84	1.20	1.02	1.08	0.84	0.48	1.02
39	—	1.74	1.14	0.42	0.60	1.62	1.74	—	—	—
48	—	—	2.86	1.92	—	2.16	0.96	—	—	—
J	—	—	2.58	1.20	—	1.62	0.42	—	—	—
Campo	0.96	0	0.12	0.24	0.11	0	2.09	0.25	0.30	0.19
B-15	—	0	0	0	—	0	0	0.05	—	0.06
Laidlaw B	—	0	0.17	0.12	—	1.62	0	0	—	0

Reaction mixture consisted of 10 μmoles ester, 10 μmoles sodium cholate, intact cells, pH 6.5, 0.06 M phosphate buffer to a total volume of 2.5 ml.
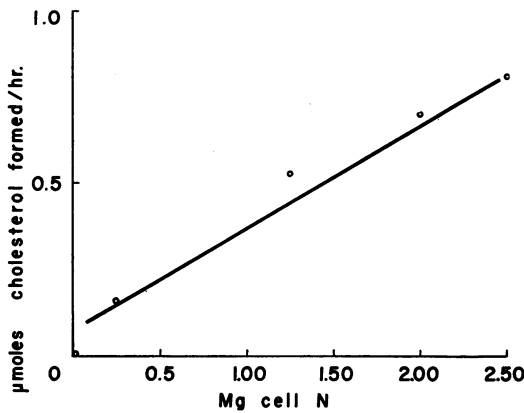
Figure 1. Effect of cellular N level on cholesterol esterase of strain O7 against cholesteryl valerate. Reaction mixture same as table 1. Reaction time, 1 hr.

TABLE 2

Stoichiometry of the cholesterol esterase reaction

	Reaction Components		
	Cholesteryl valerate	Cholesterol	Valeric acid
	μmoles/mg N		
0 Time.................	6.16	0.56	0
Final, 6 hr...........	5.05	1.68	1.02
	−1.11	+1.12	+1.02

Reaction mixture same as table 1.

TABLE 3

Cholesterol esterase activity of various cellular fractions of strain O7

Cell Fraction	Cholesteryl Ester			
	Propionate	Valerate	Caproate	Laurate
	Free cholesterol formed per mg N in 6 hr			
	μmoles			
Whole cells...........	0.95	1.50	1.63	2.75
Disrupted cells.......	1.32	1.20	1.30	1.80
Residue..............	1.06	0.95	1.32	1.90
Supernatant fluid.....	0.11	0.40	0	1.48

Reaction mixture same as table 1 except for cellular fractions indicated.

TABLE 4

Effect of pH on cholesterol esterase activity of strain O7

pH	Cholesteryl Ester				
	Propionate	Butyrate	Caproate	Caprylate	Laurate
	Free cholesterol formed per mg N in 6 hr				
			μmoles		
5.0	0.30	0	0.85	0.40	1.56
5.5	—	—	—	—	1.75
6.0	0.90	0.60	0.80	0.60	1.92
6.5	0.95	1.08	0.80	1.08	2.75
7.0	—	—	—	—	1.61
7.5	0.60	0.60	0.35	0.60	1.75
8.0	—	—	—	—	1.56
8.5	—	—	—	—	1.62
9.0	—	—	—	—	1.60

TABLE 5

Effect of surface active compounds on hydrolysis of cholesteryl valerate by strain O7

Cofactor	Free Cholesterol Formed per mg N in 6 hr
	μmoles
None.............................	1.12
Lecithin, 1 μmole..................	1.26
Lecithin, 10 μmoles...............	1.53
Lecithin, 20 μmoles...............	1.55
Na cholate, 10 μmoles.............	1.57
Na taurocholate, 10 μmoles........	1.13
Na deoxycholate, 10 μmoles.......	0.74
Na lithocholate, 10 μmoles........	0
Cephalin, 10 μmoles...............	1.49
Tween 80, 0.01%..................	0
Soap, 0.01%......................	1.34
B-II (lipoprotein), 1.0 mg.........	1.20

Reaction mixture consisted of 10 μmoles ester, cofactors as indicated, sonically treated, dialyzed cells, 0.06 M phosphate buffer, pH 6.5, to final volume of 2.5 ml.

dium cholate. The lipoprotein factor, B-II, had no effect.

The synthesis of cholesteryl esters from free cholesterol and fatty acids was demonstrated to occur with whole cells (table 6). The organisms appeared to be less capable of synthesizing cholesteryl esters than hydrolyzing them. This may be a result of the method employed, for if the

ester furnishes a means of supply of fatty acid, ester would not be expected to accumulate. This could be determined only if some means were present to block either the release of fatty acid from the ester or the oxidation of fatty acid. Further work has not been done. Sodium taurocholate was more effective than sodium cholate as a cofactor. The optimal pH for synthesis was found to be pH 6.2.

It was of interest to examine the possibility that coenzyme A might have some effect. With regard to synthesis of ester, it could be required to activate the fatty acid prior to its coupling with the sterol. Breaking of the ester bond in the presence of coenzyme A might conceivably occur by thiolytic cleavage. That coenzyme A, in conjunction with adenosine triphosphate, is an absolute requirement for synthesis of cholesteryl esters and is a stimulatory factor for the hydrolysis of ester, can be seen in table 7. This effect was noted only in Dowex treated extracts. The relatively poor activity is due probably to the small amount of enzyme present, since each mixture contained only the remainder of 1 mg whole cell N measured prior to preparation.

The possibility that an activated fatty acid was formed following hydrolysis of ester was tested by analyzing the final reaction mixture for acyl coenzyme A and by incorporating hydroxylamine into the reaction mixture. Table 8 shows that acyl coenzyme A is detectable in amounts above the cell controls. Hydroxylamine had no dele-

TABLE 6

Synthesis of cholesteryl esters by strain O7

Fatty Acid	Ester Formed per mg N in 6 hr
	μmoles
Acetic	0.30
Propionic	0.13
Butyric	0.42
Valeric	0.20
Caproic	0.42
Caprylic	0.18
Lauric	0.12

Reaction mixture consisted of 10 μmoles cholesterol, 20 μmoles fatty acid as sodium salt, 10 μmoles sodium taurocholate, intact cells, 0.06 M phosphate buffer, pH 6.2, to final volume of 2.5 ml.

TABLE 7

Effect of coenzyme A (CoA) and adenosine triphosphate (ATP) on synthesis and hydrolysis of cholesteryl esters by strain O7

Cholesteryl Ester	No Addition	CoA + ATP
	μmoles ester	
Synthesis:		
Butyrate	0	0.20
Valerate	0	0.26
Caprylate	0	0.30
	μmoles cholesterol	
Hydrolysis:		
Butyrate	0.33	0.56
Caprylate	0.55	0.90

Reaction mixtures: *Synthesis.* Cholesterol, 5 μmoles; fatty acid, Na salt, 20 μmoles; CoA, 0.075 mg; ATP, 7.5 μmoles; sodium taurocholate, 10 μmoles; Dowex 1-Cl treated sonic extract from 1 mg cell N; 0.06 M phosphate buffer, pH 6.2, to final volume of 2.5 ml. *Hydrolysis.* Cholesteryl ester, 5 μmoles; CoA, 0.075 mg; ATP, 7.5 μmoles; sodium cholate, 10 μmoles; Dowex 1-Cl treated sonic extract from 1 mg cell N; 0.06 M phosphate buffer, pH 6.5, to final volume of 2.5 ml. Reaction time: 6 hr.

TABLE 8

Detection of acyl coenzyme A following hydrolysis of cholesteryl butyrate by strain O7

Condition	Free Cholesterol	Acyl Coenzyme A or Hydroxamic Acid
	μmoles	
Hydroxylamine (5 μmoles):		
Cell control	0	0.63
Substrate control	0	0
Test	0.83	1.38
No hydroxylamine:		
Cell control	0	1.00
Substrate control	0	0
Test	0.88	1.75

Reaction mixture same as table 1.

terious but rather a stimulatory effect on the hydrolytic reaction.

Determination of the rate of the reaction with whole cells at pH 6.5 with cholesteryl butyrate, cholesteryl valerate, and cholesteryl laurate revealed similar results (figure 2). The rate curve
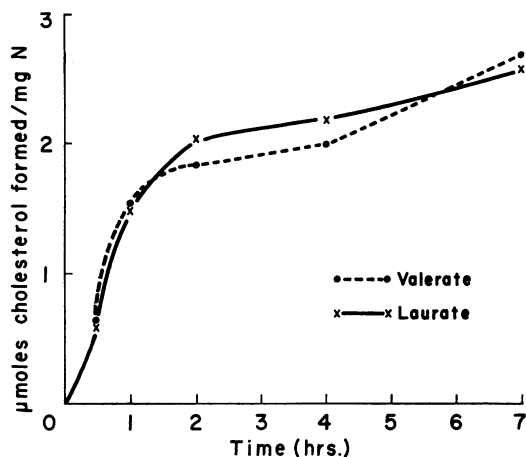
Figure 2. Rates of hydrolysis of cholesteryl valerate and cholesteryl laurate by strain O7. Reaction mixture same as table 1.

for cholesteryl butyrate was almost identical to those shown in figure 2. Maximal hydrolysis occurred in the first 2 hr, at which point the activity curves leveled. An increase in esterase activity appeared to occur between 4 and 7 hr, possibly indicating a renewed release of fatty acids. Allowing the esterase reaction to proceed at pH 7.5, the pH level at which fatty acids are known to be oxidized by these organisms, should result in no accumulation of fatty acid and in a first order type of rate curve, since rapid removal of fatty acid would tend to drive the esterase reaction toward hydrolysis. When such an experiment was conducted, no significant accumulation of free fatty acid resulted, whereas such accumulation did occur at pH 6.5. However, when the rate of the reaction was plotted as free sterol accumulation against time, no reproducible curve could be obtained. In most instances, a typical second order rather than a first order rate curve was noted. No attempt has been made yet to study the rate of accumulation of nonoxidizable analogues of fatty acids as a result of cholesterol esterase action.

No reliable results were obtained when the effect of substrate concentration was studied. Apparently, the lowest level of substrate added, 2 μmoles, was still enough to saturate the enzyme or to saturate the suspending solution. The use of less than 2 μmoles in the reaction mixture was prevented by the sensitivity of the method for determination of cholesterol and cholesteryl esters.

If cholesterol esterase, in conjunction with the other components of the lipoprotein factor, acts as a permeability mechanism for fatty acids in whole organisms, it should be expected that the esterase activity be associated with the cell wall. Disrupted cells were separated into a residue fraction (cell membrane) and a supernatant fraction (cell protoplasm). Examination of the esterase activity of these fractions revealed that in the case of every ester tested except cholesteryl laurate, almost all the activity was associated with the residue (table 3). Total activity of the residue and supernatant fluid equalled the activity of the unfractionated, disrupted cells. The activity noted in the supernatant fluid for cholesteryl laurate may be due to the enzyme becoming soluble during sonic disruption of this cell preparation.

Although the preparations used to demonstrate cholesterol esterase activity were very crude, it was of interest to determine, if possible with such preparations, whether esters of other sterols containing the 3-β-hydroxy group, aliphatic esters, and neutral fats would be hydrolyzed. Measurement of the activity was performed by titration of free fatty acid formed. As can be seen in table 9, no liberation of free fatty acid occurred when aliphatic esters were employed as substrates. Weak activity was noted toward the acetates of β-sitosterol and stigmasterol. Both these compounds and their unesterified forms possess some growth promoting capacity but do not approach the nutritional adequacy of cholesterol and its esters. However, neutral fats were readily hydrolyzed. It was necessary to determine whether the cholesterol esterase and lipase activities possessed any differences, since the possibility existed that the cholesterol esterase activity was a manifestation of a nonspecific lipase. As with the cholesterol esterase, the lipase activity was almost exclusively associated with the insoluble cell membrane and the lipase activity of Dowex 1-Cl treated disrupted cells was stimulated by addition of coenzyme A and adenosine triphosphate. On the other hand, distinct differences were noted in the stability of the two activities to heat and pH and in the inhibitory activity of various compounds. As shown in table 10, the lipase activity is more stable to heat than the esterase activity, cholestane at 10^{-4} M concentration inhibits the esterase activity completely but effects only a 60 per cent inhibition of lipase activity, and pretreatment of cells at various pH resulted in

different effects on lipase and esterase activities. Thus, it was concluded that the cholesterol esterase and the lipase were distinct.

Several possible inhibitors of cholesterol esterase activity were tested. The results can be seen in table 11. The sterols used as inhibitors were selected because they had been shown to inhibit

TABLE 9

Specificity of cholesterol esterase activity of strain O7

Ester	Free Acid Formed per mg N in 6 hr
	μmoles
Stigmasteryl acetate	0.18
β-Sitosteryl acetate	0.35
Methyl caprylate	0
Methyl oleate	0
Ethyl acetate	0
Tributyrin	7.0
Tricaprylin	6.0
Triolein	3.0
Olive oil	6.0
Peanut oil	5.0
Lard	3.0

Reaction mixture same as table 1 except for different esters.

TABLE 10

Differentiation of cholesterol esterase and lipase activities of strain O7

Condition	Cholesteryl Butyrate		Tributyrin	
	Free cholesterol	Control	Free acid	Control
	μmoles	*%*	*μmoles*	*%*
Control	1.58	100	5.0	100
Pretreatment:				
pH 3.0, 30 min	0.94	59	2.0	40
pH 5.0, 30 min	1.22	77	8.0	160
pH 9.0, 30 min	0.35	22	5.0	100
pH 11.0, 30 min	0.79	50	0	0
22 C, 15 min	1.58	100	5.0	100
37 C, 15 min	0.27	17	5.0	100
45 C, 15 min	0	0	6.0	120
55 C, 15 min	0	0	5.0	100
65 C, 15 min	0	0	3.0	60
Cholestane, 10^{-4} M	0	0	2.0	40
Cholestan-3-one, 10^{-4} M	0.92	58	2.0	40

Reaction mixture same as table 1 except for inhibitory compounds.

TABLE 11

Effect of possible inhibitors on hydrolysis of cholesteryl valerate by strain O7

Inhibitor	Free Cholesterol	Inhibition
	μmoles	*%*
None	4.10	—
Cholestane:		
10^{-3} M	0	100
10^{-4} M	0.10	98
Cholestan-3-one:		
10^{-3} M	0	100
10^{-4} M	1.00	76
Bicholesteryl ether:		
10^{-3} M	0	100
10^{-4} M	0.09	98
Estradiol:		
10^{-3}	0	100
10^{-4} M	0.02	99
2,4-Dinitrophenol, 10^{-4} M	2.18	47
Digitonin, 10^{-4} M	2.59	37

Reaction mixture same as table 1 except for addition of inhibitors.

growth (Smith and Lynn, 1958). The inhibitory action of sterols is probably directed against the cholesterol esterase, whereas the action of 2,4-dinitrophenol is undoubtedly directed against subsequent fatty acid oxidation and indirectly affects esterase activity as a result of the accumulation of unoxidized fatty acids. Digitonin, although not greatly inhibitory, could act by decreasing the solubility of sterol.

DISCUSSION

Cholesterol esterase activity of pleuropneumonialike organisms appears to be similar in nature to the cholesterol esterases present in rat liver, pancreas, and intestinal mucosa (Swell and Treadwell, 1955, Byron et al., 1953, Swell et al., 1950). Cholesterol esterase activity has not been reported previously in microorganisms. Cholesterol esterases from the mammalian tissues give optimal hydrolysis at pH 6.5, hydrolyze a great variety of fatty acid esters of cholesterol, possess rather poor capacity to esterify cholesterol, and require some bile salt as a cofactor. Differences exist between the esterases of mammalian tissues and of pleuropneumonialike organisms in that no absolute bile salt requirement can be demonstrated and no definite pattern of difference in esterase activity toward esters of short and long

chain fatty acids was noted with the microorganisms. As with the hog pancreas enzyme (Swell et al., 1953), the enzyme of pleuropneumonialike organisms possesses better esterification activity with sodium taurocholate than with sodium cholate. The major portion of esterase activity of the microorganisms was found to be associated with the particulate debris after sonic disruption. This parallels the finding of Schotz et al. (1954), who noted most of the esterase activity of rat liver in the submicroscopic particles exclusive of the nuclei and mitochondria.

Although at present no function can be excluded, the data thus far obtained appear to favor some function for the cholesterol esterase and the lipoprotein growth requirement relative to permeability.

The semipermeable membrane of microorganims is considered to be composed largely of lipoprotein (Mitchell and Moyle, 1956). Pleuropneumonialike organisms require a lipoprotein factor for growth, but they appear to incorporate it rather than to degrade it (Smith and Morton, 1951). The distribution of this lipoprotein in microorganisms is not known. The ability of protein to support growth is proportional to its ability to bind cholesterol (Smith et al., 1954). The protein may render the sterol more soluble and thus permit rapid exchange with the lipoproteins of the cell membrane.

The permeability of a metabolizable substrate has been shown to be due in several instances to some enzymatic mechanism located at the cell surface (Cohen and Monod, 1957). The data reported here indicate that the site of cholesterol esterase activity of pleuropneumonialike organisms is almost exclusively in the cellular debris following sonic lysis. Analysis of the lipid composition of the microorganisms has shown that they contain cholesterol, a higher proportion of which is in the bound form. More esterified cholesterol is found in the cellular debris (cell wall) than in the protoplasm, whereas the reverse is true for unesterified sterol (unpublished data). If the cholesterol esterase performs the function of a permease (Cohen and Monod, 1957) or translocase (Mitchell, 1957) for fatty acids, such a distribution of cholesterol esterase and of bound and free cholesterol would be expected.

The nature of the sterol and phospholipid requirements for growth and esterase activity, the ability of strains requiring the lipoprotein factor to oxidize fatty acids, and the stimulatory action

of coenzyme A and hydroxylamine on esterase activity tend to support a permeability function.

However, two observations tend to discount a permeability mechanism. It would be expected that considerably greater esterase activity would be necessary to supply fatty acid as the sole oxidizable substrate at a rate necessary for cell growth. The relatively poor esterase activity which is demonstrable by supplying cholesteryl ester may not be a true measure of total activity. The mechanism visualized for permeation of fatty acids would utilize sterol already present in the cell in soluble form by virtue of its complexing with protein. Since sterol is not degraded, the fraction of added sterol which becomes soluble and thus utilized for fatty acid transport would be dependent upon the remaining available sites on the protein and would probably be small. Demonstration of fatty acid transport through the mediation of the cholesterol esterase must be accomplished by some other method. The other discrepancy is the nonlinear rate curve for esterase activity at a pH level at which fatty acids are oxidized; however, this may be due to the method employed. Rather than attempt measurement of accumulated free cholesterol, accumulation of some nonoxidizable fatty acid analogue should be measured.

The possibility that the cholesterol esterase functions as a permease for sterol can be discounted by the fact that growth and sterol uptake occur in the absence of fatty acid (Smith and Lynn, 1958) and by the findings of Swell et al. (1958) that bile salts but not fatty acids are required for incorporation of cholesterol C^{14} in the intestinal wall of rats.

A theory can be proposed from the data obtained that the lipoprotein is either incorporated into the cell or it facilitates rapid exchange of sterol with cell lipoproteins, which act as a site for attachment or as a "solubilizer" of cholesterol. The cholesterol being neither lost nor formed is utilized by the cholesterol esterase with the cofactor participation of lecithin for synthesis of esters from activated fatty acids. The esters are then transported across the cell membrane and hydrolyzed to liberate free fatty acid or acyl coenzyme A as an oxidizable substrate. Whether the cholesterol esterase actually performs a permeability function for fatty acids and whether the lipoprotein growth requirement is intimately related to this activity must await further study.

SUMMARY

Cholesterol esterase activity toward cholesteryl esters of various fatty acids was demonstrated to be present in pleuropneumonialike organisms. Lipase activity was demonstrated and found to be distinct from cholesterol esterase activity. The strains requiring a lipoprotein growth factor possess considerably greater esterase activity than strains not requiring the growth factor. Hydrolysis of an ester results in equimolar formation of free cholesterol and fatty acid. Optimal pH for hydrolysis is 6.5 and synthesis, pH 6.2. No absolute cofactor requirement for hydrolysis was noted but surface active compounds and coenzyme A stimulate activity. Coenzyme A and taurocholate are required for synthesis. The major portion of the activity is associated with the cellular debris after sonic lysis. The reaction appears to be specific for fatty acid esters of 3-β-hydroxy $\Delta 5$ sterols. Inhibition of the hydrolysis reaction was noted with several compounds.

REFERENCES

BYRON, J. E., WOOD, W. A., AND TREADWELL, C. R. 1953 Cholesterol esterases. V. A. cholesterol esterase of rat liver. J. Biol. Chem., **205,** 483–492.

COHEN, G. N. AND MONOD, J. 1957 Bacterial permeases. Bacteriol. Revs., **21,** 169–194.

GOLDSTEIN, N. P., EPSTEIN, J. H., AND ROE, J. H. 1948 Studies of pancreatic function. IV. A simplified method for the determination of serum lipase, using aqueous tributyrin as substrate, with one hundred normal values by this method. J. Lab. Clin. Med., **33,** 1047–1051.

LIPMANN, F. AND TUTTLE, L. C. 1945 A specific micromethod for the determination of acyl phosphate. J. Biol. Chem., **159,** 21–28.

MITCHELL, P. 1957 A general theory of membrane transport from studies of bacteria. Nature, **180,** 134–136.

MITCHELL, P. AND MOYLE, J. 1956 Permeation mechanisms in bacterial membranes. Discussions Faraday Soc., **No. 21,** 1–8.

SCHOTZ, M. C., RICE, L. I., AND ALFIN-SLATER, R. B. 1954 The investigation of a cholesterol esterase in rat liver. J. Biol. Chem., **207,** 665–670.

SMITH, P. F. 1955 Amino acid metabolism by pleuropneumonialike organisms. I. General catobolism. J. Bacteriol., **70,** 552–556.

SMITH, P. F. AND LYNN, R. J. 1958 Lipid requirements for the growth of pleuropneumonialike organisms. J. Bacteriol., **76,** 264–269.

SMITH, P. F. AND MORTON, H. E. 1951 The separation and characterization of the growth factor in serum and ascitic fluid which is required by certain pleuropneumonialike organisms. J. Bacteriol., **61,** 395–405.

SMITH, P. F., LECCE, J. G., AND LYNN, R. J. 1954 A lipoprotein as a growth factor for certain pleuropneumonialike organisms. J. Bacteriol., **68,** 627–633.

SWELL, L. AND TREADWELL, C. R. 1955 Cholesterol esterases. VI. Relative specificity and activity of pancreatic cholesterol esterase. J. Biol. Chem., **212,** 141–150.

SWELL, L., BYRON, J. E., AND TREADWELL, C. R. 1950 Cholesterol esterases. IV. Cholesterol esterase of rat intestinal mucosa. J. Biol. Chem., **186,** 543–548.

SWELL, L., FIELD, H., JR., AND TREADWELL, C. R. 1953 Role of bile salts in activity of cholesterol esterase. Proc. Soc. Exptl. Biol. Med., **84,** 417–420.

SWELL, L., TROUT, E. C., JR., HOPPER, J. R., FIELD, H., JR., AND TREADWELL, C. R. 1958 Specific function of bile salts in cholesterol absorption. Proc. Soc. Exptl. Biol. Med., **98,** 174–176.

WYCOFF, H. E. AND PARSONS, J. 1957 Chromatographic microassay for cholesterol and cholesterol esters. Science, **125,** 374.