INCORPORATION OF CHOLESTEROL BY PLEUROPNEUMONIA-LIKE ORGANISMS[1]

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Cholesterol has been shown to be a growth requirement for certain pleuropneumonia-like organisms (PPLO). Sterols of slightly different molecular structure, in most instances, are either less active or inactive in supporting growth (Smith and Lynn, 1958). Those PPLO which require sterol for growth have been found to contain relatively large amounts of 3-β-hydroxy sterol (Lynn and Smith, 1960). Hence, a study was undertaken to examine the mechanism of sterol uptake and the fate of the sterol in various strains. This approach could shed light on the possible function of sterol and ultimately provide needed data for determination of the relation of PPLO to L type organisms.

MATERIALS AND METHODS

One strain (O7 of human origin) of a pleuropneumonia-like organism was used for detailed study. Two other lipoprotein requiring strains, Campo (human origin) and J (avian origin), and two nonlipoprotein requiring strains, Laidlaw B (from sewage) and B-15 (bovine origin), were used. One salt requiring L form, ADA-L derived from the β-hemolytic streptococcus, strain ADA, and Proteus 18L, an L form derived from Proteus 18 and not requiring salt or serum were also employed and were obtained from Dr. L. Dienes. *Escherichia coli* strain K-12, one diphtheroid, strain D-5 obtained from Campo cultures (Smith and Rothblat, 1960), a β-hemolytic streptococcus, ADA, a pneumococcus, and Proteus 18 comprised the bacterial species employed. Proteus 18 and its L form originated from the laboratory of R. Tulasne. The PPLO were grown and harvested as previously described (Smith, 1955). The bacterial strains and Proteus 18L were grown in 2 per cent tryptose (Difco) containing 0.5 per cent sodium chloride and 0.5 per cent glucose with a pH of 7.5 in 2- to 4-liter amounts, harvested after 18 to 24 hr by centrifugation, and washed twice with saline. The streptococcal L form, ADA-L, was grown in Albimi brucella broth containing 3 per cent sodium chloride with a pH of 7.5, harvested after 24 hr, and washed twice with 3 per cent sodium chloride.

All compounds used in this study were obtained from commercial sources (California Corporation for Biochemical Research, Distillation Products Industries, Nutritional Biochemicals Corporation, Dajac Laboratories and Chemed, Inc.). Cholesterol-4-C^{14} and Δ^4-cholesten-3-one-4-C^{14} of specific activity of 5 mc per mg were obtained from Nuclear-Chicago Corporation. Progesterone-4-C^{14} and testosterone-4-C^{14} were obtained from California Corporation for Biochemical Research.

When radioactive cells were desired as the starting material, 1.5 μc cholesterol-4-C^{14} were added as an ethanol solution to 10 liters of culture medium. The source of carrier cholesterol was PPLO serum fraction (Difco) added to give a final concentration of about 0.01 mg per ml medium. In experiments in which cells of various ages were analyzed, 4- to 6-liter cultures were harvested at 8 hr, whereas 2- to 4-liter cultures gave sufficient cell yields for greater ages.

Measurement of uptake of cholesterol during growth as a comparison to uptake by resting cells was accomplished by adding cholesterol-4-C^{14} to cultures incubated for 12 hr, i.e., at the initiation of the log phase of growth. This was necessary because not enough cells would be present for sampling 15 to 60 min after inoculation if cholesterol-4-C^{14} were added at the time

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of inoculation. In this type of experiment 1-liter cultures were harvested for each time period.

Uptake of cholesterol-4-C^{14} by resting cells was accomplished by exposing washed 40-hr-old cells of PPLO or 24-hr-old cells of bacteria and L forms to cholesterol-4-C^{14}. In the average experiment, 20 mg dry weight cells, 0.2 ml PPLO serum fraction (Difco) containing 20 mg protein and 0.2 mg cholesterol, and 0.05 μc cholesterol-4-C^{14} in ethanol were mixed to a total volume of 10 ml with 0.067 M phosphate buffer, pH 7.5. In the case of experiments on effect of pH, appropriate 0.1 M acetate or borate buffers were substituted for phosphate buffer. For the salt requiring ADA-L, buffered 3 per cent sodium chloride replaced the phosphate buffer. Samples (2 ml) were removed at 0, 15, 30, 60, and 180 min, immediately diluted to 30 ml with distilled water, or 3 per cent sodium chloride in the case of ADA-L, and centrifuged at 20,000 $\times$ g for 5 min in an SS-1A Servall centrifuge. Additional washing was found unnecessary. The sedimented cells were transferred to tared stainless steel planchets of 1 in. diameter with about 1.5 ml distilled water. After drying, the planchets were again weighed to give data for calculation of self-absorption correction.

Carbon-14 counting was performed in a Nuclear Measurements Corporation proportional counter, model PC3A, at 1750 v employing a gas mixture of 90 per cent argon and 10 per cent methane. (Matheson Company, P-10 gas). Five-minute counting periods were found to give statistically reliable results. All counts were corrected for background and to zero thickness for self-absorption. The counts recorded in this report are corrected counts:min:mg dry weight of sample. Uptake of different steroids was compared in a similar manner except the reaction mixture consisted of (per ml): ethanol-ether extracted B-II, 2 mg; lecithin 0.01 mg; carrier steroid, 0.2 mg; C^{14}-labeled steroid, 0.01 μc; and equivalent amounts of resting cells of strain O7 made up to a volume of 10 ml with 0.067 M phosphate buffer, pH 7.5.

Cells were lysed by use of a 9 kc Raytheon magneto-constriction oscillator as previously described (Smith, 1955).

Extractions and chemical analyses of the culture medium and the cells were carried out in the following manner:

Samples (20 ml) of pooled culture medium or culture supernatants were extracted three times with 1 to 2 volumes of 4:1 methylal-methanol or 2:1 chloroform-methanol. Washed cells dried with low heat under a stream of nitrogen were extracted 1 to 1½ hr in a Soxhlet extractor using 15 ml of 4:1 methylal-methanol. The extracts were dried with low heat under a stream of nitrogen. The dried residues were taken up in 5 ml N ethanolic potassium hydroxide plus 15 ml absolute ethanol and refluxed 1 hr. The saponification mixture was then transferred to a separatory funnel with 100 ml petroleum ether and 25 ml of 10 per cent sodium chloride in 0.05 N potassium hydroxide. The petroleum ether layer then was washed with 25 ml distilled water. This procedure was found to give the best recoveries of radioactivity. The petroleum ether extract was dried as stated above and the residue taken up in 1:1 acetone-ethanol. Digitonin precipitation and Liebermann-Burchard tests were made according to the methods outlined by Cook (1958). Ferric chloride tests were made according to the method of Wycoff and Parsons (1957). The supernatant fractions after digitonin precipitation were extracted with ethyl ether, washed with distilled water, dried as above, and used for ferric chloride tests and carbon-14 counting. In experiments on recovery of total nonsaponifiable lipid, samples were removed for carbon-14 counting in every step of the procedure.

Alteration of cells by use of protein end group reagents was carried out by the methods described by Smith and Boughton (1960).

The lytic action on the cells by digitonin was performed by addition of varying levels of digitonin to suspensions of cells and determining the decrease in turbidity after 1 hr at 37 C in a Klett-Summerson photometer using a 420 mμ filter.

RESULTS

There was need at the outset of this study to confirm that the sterol found in the cells of parasitic strains was derived from the cholesterol made available to the cells even though it was known that the organisms possessed an almost specific growth requirement for cholesterol. Table 1 presents data showing that both cells growing in the complete culture medium and resting cells exposed to cholesterol in the presence of the lipoprotein growth factor remove cho-

TABLE 1

Removal of cholesterol from the medium, strain O7

	Resting Cells		Growing Cells			
	Total corrected counts/min	Cholesterol: Liebermann-Burchard	Total corrected counts/min	Cholesterol		
				C^{14}	Ferric chloride	Liebermann-Burchard
		total μg			μg/ml	
Initial medium	662,000	567.3	178,200	10.7	11.4	10.0
Final medium	317,440	277.6	89,100	5.4	5.6	4.1
Per cent used	61	51	50	50	52	59

lesterol from the surrounding medium. All the methods employed give essentially the same result.

The removal of cholesterol from the medium does not warrant the conclusion that this sterol is a source of the nonsaponifiable lipids of the organisms. The data in table 2 show that essentially all the radioactivity of the whole cells is found as nonsaponifiable lipid. The values given for the saponification washings probably exceed the true values since the presence of salts results in samples of high weight necessitating a considerable self-absorption correction. There is also some likelihood of loss of small amounts of nonsaponifiable lipid in the saponification washings.

Determination of the distribution of sterol between the cell membrane (insoluble residue after sonic treatment) and the protoplasm (soluble fraction after sonic treatment) shows that approximately two-thirds to three-fourths of the sterol is found in the cell membrane (table 3). Preliminary studies indicated that the sterol in the membrane is closely associated with other cellular material, possibly protein, and can be removed only by solvent extraction, whereas the sterol in the protoplasm is free. Thus it is possible that the sterol found in the protoplasm is an artifact and the result of the method of cell breakage. The variation in the ratios of membrane to whole cell sterol (table 3) supports this possibility. The chemical method detects only digitonin-precipitable sterol while the isotope method measures all the radioactivity derived from the incorporated cholesterol.

Measurement of the uptake of cholesterol-4-C^{14} was carried out initially with both growing and resting cells. Figure 1 shows that both methods are comparable, giving the same pattern of uptake over equivalent periods of time. One difference existed in that the ratio of cholesterol-4-C^{14} to carrier cholesterol was greater in resting cell experiments than in experi-

TABLE 2

Recovery of radioactivity as nonsaponifiable lipid from strain O7

Fraction	Resting Cells		Growing Cells	
	Total corrected counts/min	Per cent recovery	Total corrected counts/min	Per cent recovery
Whole cells	380,717	100	517,625	100
Total lipid	403,560	106	586,566	113
Nonsaponifiable lipid	363,900	96	493,687	95
Saponification washings	41,125	10	46,586	9

TABLE 3

Distribution of sterol in resting or growing cells of strain O7

Expt	Digitonin Precipitable Chemical: μg/mg Dry Wt			Total Radioactive: Total Corrected Counts/min Equal No. Cells		
	Whole cell	Membrane	Membrane/whole cell	Whole cell	Membrane	Membrane/whole cells
1	12.3	8.4	0.68	30,296	22,780	0.75
2	7.0	3.8	0.54	43,493	30,467	0.70
3	18.2	13.2	0.73	40,175	25,917	0.64
4	15.3	11.4	0.75	37,651	30,181	0.80
5	9.0	6.2	0.69	10,626	8,667	0.82
6				8,540	5,746	0.68
7				7,941	6,646	0.84
8				11,317	7,142	0.63

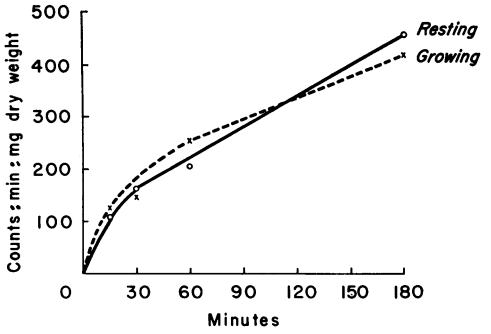

Figure 1. Comparison of uptake of cholesterol-4-C^{14} by resting and growing cells of strain O7.

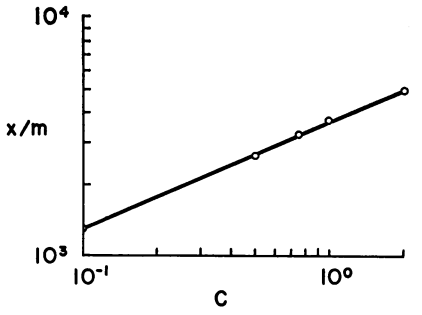

Figure 2. Adsorption of cholesterol-4-C^{14} by strain O7. Double logarithmic plot. *C* is concentration of available cholesterol in mg; *x/m* is counts:min:mg dry weight of cells. Incubated for 60 min at 37 C.

ments using growing cells. The net result is that growing cells take up more total cholesterol. However, since no significant difference was noted in the pattern of and requirements for uptake, the majority of experiments were run employing resting cells, thereby simplifying the techniques. In both cases a slow steady rate of cholesterol uptake is achieved. The values are corrected for 0 time absorption. In all experiments some radioactivity was noted in 0 time samples. In most instances this could be explained as uptake occurring during the 15 min interval of exposure in dilute solution at room temperature while the cells were being washed and sedimented.

Examination of the nature of this uptake phenomenon revealed that it was an irreversible adsorptive process. Figure 2 is a double logarithmic plot of the concentration (*c*) of available cholesterol versus the counts:min:mg dry weight or concentration of the sterol in the cells (*x/m*) employing a constant cell concentration and an adsorption time of 60 min at 37 C. This plot should yield a straight line if uptake of sterol follows the adsorption isotherm. This appears to be the case.

This adsorption is an irreversible process as shown in table 4. This table records the results of attempts to wash the radioactivity from the cells by use of lecithin which has been shown to increase the aqueous solubility of cholesterol (Smith and Boughton, 1960). When cells are exposed to cholesterol-4-C^{14} as growing cells in the complete growth medium or as resting cells in the presence of lecithin or in the presence of lecithin and the protein moiety of the lipoprotein growth factor, the adsorbed sterol cannot be removed by washing with 0.01 mg per ml solution of lecithin. This concentration of lecithin does

TABLE 4

Removal of cholesterol-4 C^{14} by washing with a solution of lecithin

No. Washing	Resting Cells Exposed to Cholesterol- 4-C^{14} Plus:			Cells Grown in 4-C^{14}-Cholesterol
	None	Lecithin	Lecithin-protein	
	counts:min:mg dry wt			*counts:min:mg dry wt*
1	1,868	1,345	855	711
2	1,464	1,211	820	757
3	1,322	1,326	852	752

not induce lysis of the cells. One exception is noted—when resting cells are exposed to cholesterol alone. The reduction of radioactivity noted is probably due to solubilization in the lecithin wash solution of unadsorbed precipitated cholesterol. If resting cells labeled by growing in the presence of cholesterol-4-C^{14} are allowed to incubate with unlabeled cholesterol together with lecithin and the above mentioned protein, no decrease in the radioactivity of the cells nor any appearance of radioactivity in the supernatant solution is noted. This was found to be true with cells 24 and 48 hr old. Although the cells are unable to release or exchange sterol, they still possess the capacity for additional sterol uptake.

The capacity to adsorb sterol irreversibly was found not to be a property specific for parasitic strains (those which require sterol for growth). A previous report (Lynn and Smith, 1960) stated that the saprophytic strains contained no 3-β-hydroxy sterol detectable by digitonin

precipitation or Liebermann-Burchard reaction and no sterol giving a positive reaction on paper with antimony pentachloride or *m*-dinitrobenzene (ketosteroids). The radioactivity derived from cholesterol-4-C^{14} in the saprophytic strains can be recovered in the nonsaponifiable lipid fraction as is the case with parasitic strains (table 2). In addition considerable amounts of nonsaponifiable lipid can be found in saprophytic strains grown in lipid-free medium. It seems likely that the cholesterol taken up by the saprophytic strains is converted into some nonsaponifiable lipid unreactive with digitonin and the Liebermann-Burchard reagent. This nonsaponifiable lipid fraction gives a positive ferric chloride test. On the other hand the parasitic strains incorporate cholesterol in a form which at least in part possesses some of the structural properties of the cholesterol molecule. Table 5 shows that all strains of PPLO tested possessed a similar capacity for cholesterol uptake. In contrast are the bacterial strains examined which take up much smaller amounts of cholesterol. Of the L forms, the Proteus 18L behaves as PPLO but with significantly less total uptake, whereas ADA-L behaves as the bacteria. Thus the ability to adsorb sterol in large amount appears to be a property common and rather specific for PPLO in general. Recently, it has been reported by Hartman and Holmlund (1960) that β-sitosterol and cholesterol are adsorbed by resting cells of *Penicillium canescens*, other fungi, and two bacterial cultures.

The effects of various environmental conditions on the uptake of cholesterol-4-C^{14} are those expected of an adsorption process. Thus increase of temperature results in an increase of adsorption (table 6). The amount of sterol adsorbed is related to the cell concentration when the sterol concentration remains constant (table 7). When the adsorption is carried out at varying pH, significant cholesterol uptake occurs at pH levels below and above the isoelectric point of the lipoprotein supplying the cholesterol (pH 5.2) and of the cells (pH 6) (table 8). No distinction could be made as to whether the effect was due to the absence of a charge on the lipoprotein or on the cells. The charge on the cells is probably more important since cholesterol is taken up by the cells in the absence of the protein (Smith and Boughton, 1960). Controls without cells were run to estimate the amount of sterol

TABLE 5

Comparison of cholesterol-4-C^{14} uptake by resting cells of PPLO, L forms and bacteria

Organism	Time (Corrected for 0 Time) (min)				Uncorrected 0 Time
	15	30	60	180	
	counts:min:mg dry wt				*counts:min:mg dry wt*
PPLO:					
O7	177	289	427	873	78
Campo	159	365	580	904	70
J	108	235	537	925	153
Laidlaw B	292	460	698	1402	152
B-15	59	143	470	948	110
L forms:					
Proteus 18L	51	112	201	278	50
ADA-L	36	20	38	89	78
Bacteria:					
Escherichia coli, K-12	71	66	15	234	118
Pneumococcus	65	63	98		59
Proteus 18	251	262	280	313	199
Streptococcus, ADA	0	0	42		71
Diphtheroid, D-5	133	176	197	233	86

TABLE 6

Effect of temperature on uptake of cholesterol-4-C^{14} by resting cells of strain O7

Temperature	Uncorrected 0 Time	Uptake after 180 min (Corrected for 0 Time)
C	*counts:min:mg dry wt*	
0	75	34
22	78	231
37	76	369
50	73	767

TABLE 7

Effect of cell concentration on uptake of cholesterol-4-C^{14} by resting cells of strain O7

Dry Wt Cells	Uncorrected 0 Time	Uptake after 180 min (Corrected for 0 Time)
mg	*counts:min*	
0	56	0
0.7	285	1062
1.5	482	1937
2.8	660	2959
5.0	1003	3866

precipitated from the mixture, thus not being available for adsorption. Only at pH 5 was considerable precipitation observed. Thus in addition to maintaining a charge on the cells or the protein, pH exerts an effect on the solubility of the exogenous cholesterol.

An attempt was made to saturate the cells with cholesterol. As seen in table 9 saturation was still incomplete after 8 hr exposure of resting cells. However, the rate of uptake diminishes with increased time. Cells grown in a great excess of cholesterol (0.2 mg per ml culture medium) exhibited a lessening capacity for adsorption (table 10).

The fact that the cells could not be saturated with cholesterol raised doubt that other sterols might compete with cholesterol in the adsorption process. When Δ^4-cholesten-3-one-4-C^{14} was substituted for cholesterol-4-C^{14} the pattern and the degree of uptake was almost identical to cholesterol (table 11). Cholesterol was found to have a slight stimulatory effect on uptake of Δ^4-cholesten-3-one, whereas Δ^4-cholesten-3-one had a slight inhibitory effect on cholesterol uptake (table 12). The significance of difference in these values is questionable. These results

TABLE 8

Effect of pH on uptake of cholesterol-4-C^{14} by resting cells of strain O7

pH	Uptake after 180 min		Precipitation of Added Sterol
	Uncorrected	Corrected for 0 time adsorption	
	counts:min:mg dry wt cells		%
3.0	298	217	0
4.0		371	0
5.0	150	38	50
5.3	109	0	2
5.6	157	80	5
6.0	383	220	0
7.5	504	457	0
9.0	481	360	0
11.0	426	288	0

TABLE 9

Effect of time on uptake of cholesterol-4-C^{14} by resting cells of strain O7

Time	Uncorrected	Corrected
	counts:min:mg dry wt	
0	111	0
15 min	346	235
30 min	521	410
1 hr	762	651
3 hr	1138	1027
5 hr	1588	1477
6½ hr	1872	1761
8 hr	2077	1966

TABLE 10

Effect of possible inhibitors on uptake of cholesterol-4-C^{14} by resting cells of strain O7

Compound or Condition	Amount	Uptake after 180 min	
		Uncorrected 0 time	Corrected for 0 time
	M	*counts: min:mg dry wt*	
None		73	639
Boiled cells		82	857
Cholestane	10^{-3}	130	493
Cholestan-3-one	10^{-3}	112	868
2,4-Dinitrophenol	10^{-3}	75	1099
Sodium azide	10^{-3}	76	821
Digitonin	10^{-3}	144	751
Cells grown in excess of cholesterol		77	484
Cells aged 7 days at 37 C		84	792
Ethanol-ether extracted cells		70	125
1-Fluoro-2,4 dinitrobenzene treated cells		111	950
p-Chloromercuribenzoate treated cells		81	755
Potassium periodate treated cells		80	755

TABLE 11

Comparison of uptake of various 4-C^{14}-labeled steroids by strain O7

Steroid	Time (min)				Uncorrected 0 Time
	15	30	60	180	
	counts:min:mg dry wt				*counts:min: mg dry wt*
Cholesterol-4-C^{14}	202	550	595	1299	529
Δ^4-Cholesten-3-one-4-C^{14}	556	602	725	922	475
Progesterone-4-C^{14}	15	13	22	27	35
Testosterone-4-C^{14}	0	0	0	11	0

TABLE 12

Effect of Δ^4-cholesten-3-one on uptake of cholesterol by resting cells of strain O7

Sterol for which Uptake Measured*	Other Nonlabeled Sterol Added		Uncorrected 0 Time	Uptake after 180 min
		mg/ml	*counts : min : mg dry wt*	*counts : min : mg dry wt*
Cholesterol-4-C^{14}	None		111	1,027
	Δ^4-Cholesten-3-one	0.01	126	1,161
		0.10	128	850
Δ^4-Cholesten-3-one-4-C^{14}	None		272	928
	Cholesterol	0.005	227	1,083
		0.01	260	1,120
		0.05	261	1,197
		0.10	290	1,259

* Final concentration of sterol measured 0.01 mg per ml.

indicate that the specificity of the 3-β-hydroxy group is not associated with the process of initial adsorption of sterol by the cells.

Since Δ^4-cholesten-3-one had never been assayed for its ability to substitute for cholesterol in the growth of PPLO (Smith and Lynn, 1958), it is significant in relation to the uptake of this compound to report on its growth inhibitory action. Figure 3, curve A, shows that increasing amounts of Δ^4-cholesten-3-one added to the complete growth medium exert a growth inhibitory action. When increasing amounts of cholesterol are added to the growth medium containing 0.05 mg per ml Δ^4-cholesten-3-one, only partial relief of growth inhibition is obtained (curve B).

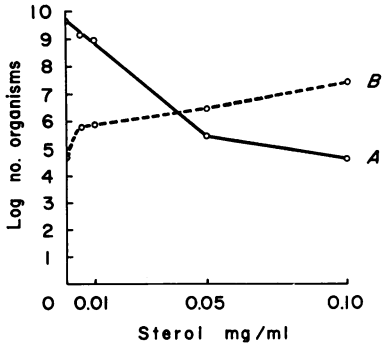

Figure 3. Effect of Δ^4-cholesten-3-one on growth of strain O7. *Curve A* shows inhibition of growth by increasing amounts of Δ^4-cholesten-3-one in presence of 0.01 mg per ml cholesterol. *Curve B* shows partial relief of growth inhibition induced by 0.05 mg per ml Δ^4-cholesten-3-one by increasing amounts of cholesterol.

Additional attempts to prevent adsorption of cholesterol-4-C^{14} by resting cells met with failure (table 10). Heating of cells, treatment of cells with protein end group reagents, aging of cells, and the addition of various possible inhibitory compounds had no significant effect. The noninhibitory action of respiratory poisons would indicate that the initial uptake is an inactive process requiring no energy. Removal of the lipid from the cells by ethanol-ether extraction, admittedly a harsh process, did reduce significantly the amount of sterol adsorbed. A comparison was made of the ability of strain O7 to adsorb various 4-C^{14}-labeled steroids. Δ^4-Cholesten-3-one is the 3-keto analogue of cholesterol. Testosterone and progesterone differ from Δ^4-cholesten-3-one in that the side chain has been removed or shortened. Δ^4-Cholesten-3-one possessing the side chain of cholesterol is adsorbed similarly to cholesterol. The two compounds lacking the side chain are not adsorbed (table 12). These results indicate that the side chain is specifically required for adsorption onto the cells.

The presence of the major portion of nonsaponifiable lipid in the cell membranes of PPLO, the continuous uptake of sterol with time, and the inability to alter the adsorptive process except by interference with the lipids of the cells suggested that lipids may constitute a very important if not the major structural entity of the cell. In this connection it was of interest to compare the sensitivity of PPLO and bacteria to digitonin. Table 13 presents the results of experiments on the lysis of various organisms by digitonin. None of the bacterial

TABLE 13

Effect of digitonin on resting cells of PPLO, L forms, and bacteria

Organism*	Klett Reading with Digitonin at (mg/ml):				
	0	0.01	0.02	0.1	0.2
PPLO					
O7	135	97	86	64	58
J	195	195	195	176	164
Laidlaw B:					
Grown with sterol	290	290	268	163	162
Grown without sterol	290	284	284	250	236
L forms					
Proteus 18L:					
Grown with sterol	455	430	430	425	415
Grown without sterol	345	342	342	337	330
ADA-L	500	500	500	500	500
Bacteria					
Escherichia coli K-12	186	186	186	181	186
Pneumococcus:					
Grown with sterol	171	171	164	143	130
Grown without sterol	190	190	193	190	187
Proteus 18:					
Grown with sterol	360	360	360	360	370
Grown without sterol	294	305	300	305	305
Streptococcus, ADA	212	212	212	212	218
Diphtheroid, D-5	241	242	242	248	246

* Grown in presence of cholesterol unless stated otherwise.

strains except the pneumococcus were affected. Pneumococci have been reported to be lysed by saponin (Klein and Stone, 1931; Klein, 1935). The parasitic strains of PPLO are susceptible to lysis. Laidlaw B, a saprophytic strain, grown in the presence of cholesterol exhibits considerably more sensitivity to digitonin lysis than when grown in the absence of cholesterol. Exposure of resting cells of PPLO to additional cholesterol prior to exposure to digitonin had no effect on the lytic activity of digitonin. Of the two L forms tested, the salt requiring ADA-L was insusceptible, whereas the Proteus 18L was lysed only to a slight degree.

CONCLUSIONS

Incorporation of sterol in major part into the cell membrane fraction appears to be a capacity peculiar to PPLO. Parasitic and saprophytic strains both possess this ability but are distinguishable by the change in nature of the sterol following incorporation. Saprophytic strains further appear capable of synthesizing their own nonsaponifiable lipid. One L type organism, Proteus 18L, adsorbs cholesterol in a pattern similar to PPLO but to a much lesser extent. The salt requiring, osmotically unstable ADA-L behaves as the bacteria with regard to sterol uptake both in pattern and extent of uptake. It is possible that an examination of more osmotically stable L type organisms would show some behaving more typically of PPLO than Proteus 18L.

The initial step in the process of sterol metabolism by PPLO appears to be an irreversible adsorption onto the cell membrane. This step is an inactive one, physiologically, requiring no enzyme and no energy. It behaves as a typical physical process. However, there are requirements if an orderly and sustained adsorption, necessary for growth, is to be achieved. These requirements are the presence of a protein possessing certain physical properties, an environment favorable to acquisition of a charge on protein or cell, and a source of water soluble sterol (Smith and Boughton, 1960).

The side chain specificity of the sterol required for growth can be explained as a need for that portion of the molecule adsorbed onto the cell. Sterols with side chains differing from cholesterol either have a limited or no ability to support growth (Smith and Lynn, 1958) and cannot be adsorbed onto the cells.

Practically all efforts to gain some insight as to the site of attachment of the sterol in the cell membrane have failed. It was concluded that a charge on the cell is necessary for the sterol to be adsorbed, that once adsorbed the sterol can be removed only by drastic procedures such as solvent extraction, that rather large amounts of sterol are adsorbed and the lipid fraction of the cell, possibly phospholipid, plays some role in this process.

Following adsorption, the sterol appears to meet a fate dependent upon the type of PPLO employed. In both parasitic and saprophytic strains the supplied sterol resides in the nonsaponifiable lipid fraction of the cell membrane. However, in the parasitic strains, the sterol retains some of the identity of cholesterol as evidenced by the presence of a 3-β-hydroxy group thereby being precipitable with digitonin (Lynn and Smith, 1960). On the other hand, saprophytic strains, whether allowed to syn-

thesize their own nonsaponifiable lipid or supplied cholesterol as the starting sterol, appear to possess a sterol quite different from cholesterol, that is, one apparently nonprecipitable with digitonin and unreactive with the Liebermann-Burchard reagent yet reactive with the nonspecific ferric chloride reagent. These conclusions are further verified by the finding that parasitic strains are extremely sensitive to digitonin, whereas saprophytic strains even when grown in the presence of cholesterol are much less sensitive to digitonin induced lysis.

What function the sterol plays in the cells is still unclear. The copious amounts found in the cell suggest a structural role. In addition, the major portion of sterol is found in the cell membrane, no energy is required for the process to occur indicating no active transport across the cell membrane and the cells are susceptible to lysis by the cholesterol complexing saponin, digitonin.

On the other hand, the freedom of 3-β-hydroxy site, the specificity of the 3-β-hydroxy group for growth (Smith and Lynn, 1958) and its nonrequirement for initial adsorption, and the cholesterol esterase activity of the cells (Smith, 1959) suggest an additional metabolic function particularly for parasitic strains (Smith, 1960).

SUMMARY

Cholesterol-4-C^{14} is removed from the supernatant medium by resting and growing cells of pleuropneumonia-like organisms. This sterol was found to be incorporated solely in the nonsaponifiable lipid fraction, the major portion of which is located in the cell membrane. This distribution of nonsaponifiable lipid was further verified by the lytic activity of digitonin on the cells containing sterol. The sterol is taken up by an irreversible adsorption process and can be removed only by solvent extraction. The initial adsorption process is affected by time, temperature, cell concentration, and pH, but not by respiratory poisons and destruction of protein end groups on the cell surface. Only steroids possessing a side chain similar to cholesterol are adsorbed, indicating that the adsorption of the steroid occurs through the 8 carbon side chain. The uptake of sterol is relatively specific for pleuropneumonia-like organisms and some L forms. Some bacteria and osmotically labile L forms also adsorb sterol but to a lesser degree.

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