# ROLE OF PROTEIN AND PHOSPHOLIPID IN THE GROWTH OF PLEUROPNEUMONIA-LIKE ORGANISMS[1]

PAUL F. SMITH AND JANET E. BOUGHTON[2]

*Department of Microbiology, School of Medicine, University of Pennsylvania, and Department of Microbiology, Temple University School of Medicine, Philadelphia, Pennsylvania*

Received for publication May 23, 1960

The cholesterol component of the lipoprotein required for growth of certain pleuropneumonia-like organisms (PPLO) has been described as the actual compound utilized by these organisms (Smith and Rothblat, 1960). That the two other components, protein and phospholipid, are necessary factors in media which support growth has been reported by several investigators (Edward and Fitzgerald, 1951; Rodwell, 1956; Smith, Lecce, and Lynn, 1954; Smith and Lynn, 1958). Both active and passive roles have been postulated for these two components (Rodwell, 1956; Smith, 1960). The results presented here represent a study of the possible functions of the protein and phospholipid with the conclusions that both play an essentially passive role in the growth and physiology of these organisms.

## MATERIALS AND METHODS

Three strains, O7 (human), J (avian origin), and Laidlaw B (from sewage), were used. Quantitative growth assays were carried out as previously described (Smith and Lynn, 1958). Large volume cultures were grown and harvested as has been reported (Smith, 1955). The proteins used in this study were obtained from Nutritional Biochemicals Corporation except in the case of B-II, which was prepared as described by Smith et al. (1954). Proteins were rendered lipid-free by alcohol-ether (3:2) extraction of the dried material, made up in neutral aqueous solution, and filtered through Selas O2 filter candles.

Alterations of the B-II protein and fraction V bovine albumin so as to destroy or block end groups were carried out as follows: amino groups by reaction with 1-fluoro-2,4-dinitrobenzene (Fraenkel-Conrat, Harris, and Levy, 1955), with the sodium salt of 2,4-dinitrobenzene sulfonate (Eisen, Belman, and Carsten, 1953), by acetylation (duVigneaud and Meyer, 1932) and by reaction with carbobenzoxy chloride (Bergmann and Zervas, 1932); sulfhydryl groups by iodination (Pressman and Eisen, 1950), reaction with mercuric chloride, monoiodoacetic acid, and *p*-chloromercuribenzoate (Chinard and Hellerman, 1954); hydroxyl groups by periodate oxidation (Desnuelle, 1953). The dinitrobenzene compounds were obtained from Distillation Products Industries and the carbobenzoxy chloride and *p*-chloromercuribenzoate from Dajac Laboratories, Philadelphia. Following dialysis from 24 to 48 hr against distilled water, the altered proteins were filtered through Selas O2 candles and incorporated into the basal medium or used in cholesterol uptake experiments.

The complete growth medium for protein studies consisted of the appropriate protein at varying levels, cholesterol, 0.01 mg per ml, lecithin, 0.005 mg per ml, and ether extracted PPLO broth; for phospholipid studies it consisted of lipid-free B-II, 1 mg per ml, cholesterol, 0.01 mg per ml, the appropriate levels of phospholipid, fatty acid or surface active agent, and ether extracted PPLO broth (Smith and Lynn, 1958). Cholesterol and lecithin were added as ethanol solutions, whereas fatty acids and surface active agents were added as autoclaved or filtered neutral aqueous solutions.

Experiments in which growth inhibition and its relief were being examined made use of unextracted PPLO broth supplemented with serum fraction (Morton, Smith, and Leberman, 1951; Smith and Morton, 1951).

Fatty acids, phospholipids, and surface active

[1] The work reported in the paper was supported in part by research grant E-2179 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, and by contract Nonr-551(31) between the Office of Naval Research and the University of Pennsylvania.

[2] Present address: Institute of Microbiology, Rutgers, The State University of New Jersey, New Brunswick, New Jersey.

agents were obtained from Distillation Product Industries, California Corporation for Biochemical Research, and Nutritional Biochemicals Corporation. The cholesterol used in these experiments was recrystallized twice from glacial acetic acid. Cholesterol-4-$C^{14}$ with specific activity of 5 mc per millimole was obtained from Nuclear-Chicago Corporation. Sodium oleate-1-$C^{14}$ of specific activity of 2 mc per millimole obtained from the California Corporation for Biochemical Research.

Radioactive measurements were made as described previously (Smith and Rothblat, 1960). The counts recorded in this paper represent corrected counts:min:mg of dry weight of sample of whole cells. Cholesterol uptake experiments were performed as described by Smith and Rothblat (1960). Since the exact ratio of cholesterol-4-$C^{14}$ to unlabeled cholesterol was not maintained in each experiment, all comparisons are made in reference to the control. Tests to determine the aqueous solubility of cholesterol were performed as follows. In a total volume of 10 ml of pH 7.5, 0.067 M phosphate buffer containing 0.01 mg per ml cholesterol and approximately 3 m$\mu$c per ml of cholesterol-4-$C^{14}$, was contained varying levels of phospholipids, fatty acids, and surface active agents or proteins. After 1 hr in a 37 C water bath, a 2-ml sample of the mixed suspension was placed in a planchet and dried. The remainder was centrifuged at 20,000 $\times$ $g$ for 10 min in a Servall angle centrifuge and 2 ml of the supernatant fluid placed in a planchet and dried. The ratio of corrected counts per min of the suspension and the supernatant fluid served as a measure of the aqueous solubility of cholesterol.

The lytic activity of various phospholipids, fatty acids, and surface active agents was tested by incorporating varying levels of lytic agent with a suspension of organisms. Appropriate cell and lytic agent controls were run. After 3 hr incubation at 37 C, turbidity measurements were made in the Klett-Summerson colorimeter, using a 420 m$\mu$ filter. Antilytic activity of proteins was measured in the same way except for the added presence of the antilytic agent and its proper control.

Protein degradation was measured by increase of free amino groups (formal titration) and by loss from the supernatant medium using the method of Stadtman, Novelli, and Lipmann (1951) for protein determinations. Phospholipid degradation was measured by release of inorganic phosphate (Fiske and SubbaRow, 1925) and by release of free fatty acids (Goldstein, Epstein, and Roe, 1948).

Uptake or adsorption of surface active agents was limited to the only available type of $C^{14}$-labeled compound capable of supporting growth, i.e., sodium oleate. Uptake was measured with both growing and resting cells. In the case of growing cells, 1 $\mu$c of sodium oleate in pH 7.5, 0.067 M phosphate buffer plus enough unlabeled sodium oleate to give a level of 0.001 mg per ml was added to 4 liters of PPLO medium supplemented with 1 per cent PPLO serum fraction and inoculated with strains O7, J, and Laidlaw B. After 48 hr incubation, the cells were harvested, washed twice with distilled water, placed in planchets, dried, and counted. In the case of resting cells, washed cells of strains O7, J, and Laidlaw B were exposed to 0.001 mg per ml of oleate containing about 0.2 $\mu$c of sodium oleate-1-$C^{14}$ per 10 ml and 1 mg per ml of PPLO serum fraction as a source of protein and cholesterol. Samples (2 ml) were removed at 0, 15, 30, 60, and 180 min, washed with distilled water, and dried on planchets for counting.

Uptake or adsorption of protein was studied using 1-fluoro-2,4-dinitrobenzene derivatives of B-II, fraction V bovine albumin, and PPLO serum fraction. Experiments were conducted only with growing cells of strain O7 in 200-ml and 2-liter cultures. To the basal medium 1 mg per ml of a given protein was added together with 0.01 mg per ml cholesterol and 0.005 mg per ml lecithin in place of the PPLO serum fraction. The cells were harvested after 48 hr, washed three times with saline, and observed visually for a yellow coloration. The cells were then suspended in 5 ml distilled water to which was added 0.1 mg per ml sodium oleate to effect lysis. The absorption spectra of the lysates were determined in a Beckman spectrophotometer and compared with the absorption spectra of the 1-fluoro-2,4-dinitrobenzene derivatives of B-II and fraction V albumin.

## RESULTS

Several possible functions for the protein requirement can be postulated. These include a) a protective effect for the organisms against toxic growth requirements, such as long-chained

unsaturated fatty acids, and against toxic end products of metabolism, b) a source of intact protein or a peptide unable to be synthesized by the organisms, c) a water soluble attachment for water insoluble lipids required for growth, and d) a regulatory or feeding source of water insoluble lipids required for growth. All of these possible functions were examined in an effort to establish the most probable action of the protein requirement.

The procedure by which some function might be elucidated consisted of correlating the abilities of proteins to support growth with their capabilities in performing some other activity associated with the physiology of the organisms. Thus, the initial step in this study was the quantitation of the growth response of the organisms to many varied proteins (table 1). Growth response is tabulated in terms of a control which contained no protein. The level of protein given in the table represents that amount which permitted the best growth response. Of the purified proteins, B-II and $\beta$-lactoglobulin gave a significant response. Of the impure preparations, exclusive of whole serum and the PPLO serum fraction which contain B-II, only fraction V bovine albumin gave a measurable response. Of interest is the fact that purification of the albumin to its crystalline state, resulted in loss of the growth promoting activity. It was concluded that the active component in fraction V was a contaminant.

It was previously reported (Smith and Morton, 1951) that protein disappeared from the medium during growth of these organisms. Repeated attempts, employing the trichloroacetic acid method of Stadtman et al. (1951), to measure the disappearance of B-II during growth of strain O7, have indicated that a small but definite amount is lost from the culture supernatant. The exact quantitative amount varies from experiment to experiment but usually is from 0.1 to 0.3 mg per ml. This disappearance of protein is attendant with a disappearance of small amounts of amino groups as judged by formal titration. Since no increase in free amino groups was noted it was assumed that the protein was not hydrolyzed. Although this type of experiment is indicative of uptake or adsorption of protein by the cells or of precipitation of protein in the medium, it does not distinguish between these possibilities. Another approach

TABLE 1

*Ability of proteins to support growth of strain O7*

| Protein | Optimal Level Protein | Growth Response |
|---|---|---|
| | *mg/ml* | |
| None | 0 | 1.00 |
| B-II | 1.0 | 1333.0 |
| PPLO serum fraction (Difco) | 2.0 | 50.0 |
| Whole bovine serum | 3.1 | 27.8 |
| $\beta$-Lactoglobulin | 5.0 | 19.3 |
| Fraction V bovine albumin | 5.0 | 11.2 |
| Fraction III bovine $\beta$-globulin | 0.1 | 3.13 |
| Bovine hemoglobin | 0.5 | 2.63 |
| Crystalline bovine albumin | 1.0 | 2.13 |
| Fraction II bovine $\gamma$-globulin | 1.0 | 0.90 |
| Fraction IV bovine $\alpha$-globulin | 0.1 | 0.82 |
| Bovine glycoprotein | 1.0 | 0.27 |
| Bovine globin | 0.1 | 0.04 |

was attempted to clarify the reason for the disappearance of protein. As will be discussed later, reaction of the amino groups of B-II and fraction V albumin with 1-fluoro-2,4-dinitrobenzene did not significantly affect their abilities to support growth. This labeling resulted in a firm chemical bond and gave a compound easily detected in minute amounts (approximately 10 $\mu$g) by spectrophotometry. The absorption peak of the 1-fluoro-2,4-dinitrobenzene labeled proteins was found to be 356 m$\mu$. Cells harvested from 200-ml cultures, in which the protein was replaced by 1-fluoro-2,4-dinitrobenzene labeled B-II or fraction V, were found to contain no detectable labeled protein, i.e., <0.01 mg per 0.5 mg cell protein, in either the cell membranes or protoplasm. Cells harvested from a 2.2-liter culture to which 1-fluoro-2,4-dinitrobenzene labeled PPLO serum fraction was added likewise were found to contain no detectable labeled protein, i.e., <0.01 mg per 16.4 mg protein. Thus the disappearance of protein from the supernatant culture medium probably was due to precipitation of protein during growth. Upon repeated washing of the cells, it was possible to free them of it.

Surface active agents have been shown to have an adverse effect on these organisms (Smith and Sasaki, 1958). Surface active agents are also growth requirements for these organisms (Smith and Lynn, 1958). Thus an obvious function for a protein could be to protect the organisms from

the adverse action of these compounds. Rodwell (1956) has shown that protein and cholesterol will prevent the adverse effects of sodium oleate on the bovine pleuropneumonia organism. Figure 1 shows the lytic activity of different types of surface active agents. Those compounds tested but not represented in the figure can be classed as one of two types. Cephalin, sodium cholate, and sodium stearate are equivalent to Tween 80 (polyoxyethylene sorbitan), and sodium linoleate and sodium linolenate are almost identical in lytic activity to sodium oleate. Figure 2 presents the effect of various types of proteins on prevention of lysis induced either by 0.01 mg per ml of sodium oleate or by 0.1 mg per ml of lecithin. No prevention of lysis was noted at any level of protein added (maximum of 2 mg per ml) when 0.1 mg per ml of sodium oleate was used to induce lysis. The use of larger amounts of protein resulted in turbidity due to proteins. Among the proteins tested, fraction V bovine albumin, crystalline bovine hemoglobin and $\beta$-lactoglobulin behaved as B-II and crystalline bovine albumin, i.e., prevented lysis at about 1 mg per ml. Bovine glycoprotein behaved similarly to fraction II bovine $\gamma$-globulin. The major point of interest with these results is that although all proteins active in supporting growth possess the capacity to prevent lysis, some proteins inactive for growth also prevent lysis. Thus there appears to be no good correlation between the property of lysis prevention and growth promotion.

Presumably the inhibitory activities of surface active agents on growth is due to their lytic properties. It was found that 0.01 mg per ml of sodium oleate would completely inhibit growth in the complete medium when an inoculum of

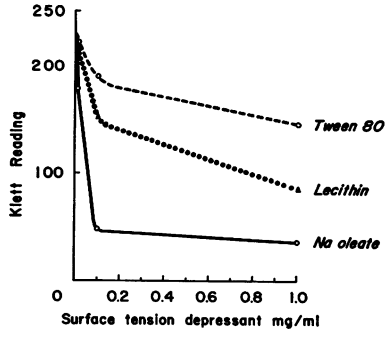


*Figure 1.* Lytic activity of various types of surface active agents.

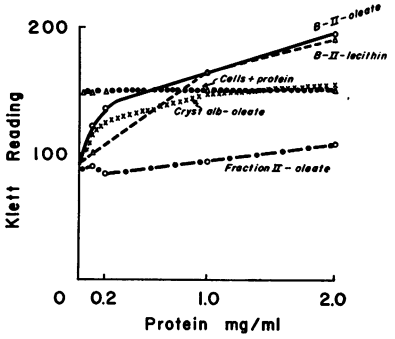


*Figure 2.* Prevention of lysis induced by sodium oleate and lecithin by various proteins.

$10^5$ cells per 10 ml culture was used. In table 2 it can be seen that the pattern of relief by various proteins of oleate and lecithin induced growth inhibition is similar to that of lysis prevention. Proteins both active and inactive for growth will negate the growth inhibitory activity of surface active agents.

Previous data (Smith et al., 1954) indicated that some correlation existed between the ability of a protein to support growth and its sterol binding capacity. Although these results were of a preliminary nature and firm binding of sterol may not have occurred, it has been found by the use of $C^{14}$-labeled cholesterol that proteins active for growth possess a capacity to increase the aqueous solubility of cholesterol. Table 3 lists the level of lipid-free protein in pH 7.5, 0.067 M phosphate buffer and the per cent of soluble cholesterol. These results are not striking enough to account for the major role of protein in the growth of PPLO.

One property of proteins which appeared to possess such a striking correlation to growth promotion is the regulation of cholesterol uptake. As noted in a previous paper (Smith and Rothblat, 1960) the patterns of uptake with growing and resting cells are identical. By necessity (some proteins fail to support growth) resting cells were used to study the effect of proteins on cholesterol uptake. The proteins tested could be grouped into three classes with regard to both growth promotion and regulation of cholesterol uptake, i.e., a) those which permitted optimal growth and permitted a slow, steady and lengthy uptake of cholesterol, b) those which stimulated some growth and permitted a more rapid and high total uptake, and c) those which did not support growth and actually inhibited

TABLE 2

*Prevention of sodium oleate and lecithin induced growth inhibition by proteins, strain O7*

| Protein Added | Protein Concn | Growth Response | |
|---|---|---|---|
| | | Sodium oleate 0.01 mg/ml | Lecithin 0.1 mg/ml |
| | *mg/ml* | | |
| None | 0 | 0 | $2.3 \times 10^6$ |
| Crystalline bovine albumin | 0.5 | $5.0 \times 10^3$ | |
| | 2.5 | $4.7 \times 10^8$ | |
| | 5.0 | $4.5 \times 10^9$ | |
| Fraction V bovine albumin | 0.5 | $2.3 \times 10^4$ | |
| | 5.0 | $4.6 \times 10^8$ | |
| | 10.0 | $4.2 \times 10^8$ | |
| B-II | 0.5 | $7.5 \times 10^4$ | $5.8 \times 10^8$ |
| | 2.5 | $3.2 \times 10^9$ | $8.1 \times 10^8$ |
| | 5.0 | $5.3 \times 10^9$ | $6.5 \times 10^8$ |
| Fraction II bovine $\gamma$-globulin | 0.5 | 0 | $2.5 \times 10^6$ |
| | 2.5 | 0 | $3.6 \times 10^6$ |
| | 5.0 | 0 | $2.6 \times 10^6$ |
| | 10.0 | 0 | 0 |
| $\beta$-Lactoglobulin | 0.5 | 0 | |
| | 2.5 | $2.1 \times 10^8$ | |
| | 5.0 | $1.9 \times 10^7$ | |
| Bovine glycoprotein | 2.5 | 0 | |
| | 5.0 | $1.5 \times 10^7$ | |
| | 10.0 | $1.2 \times 10^7$ | |
| No inhibitor | | $5.0 \times 10^9$ | |

TABLE 3

*Effect of proteins on aqueous solubility of cholesterol*

| Protein | Protein Concn | Soluble Cholesterol |
|---|---|---|
| | *mg/ml* | % |
| *Experiment A* | | |
| None | 0 | 12 |
| B-II | 0.5 | 26 |
| | 1.0 | 22 |
| | 2.5 | 20 |
| | 5.0 | 19 |
| Fraction V albumin | 0.5 | 20 |
| | 1.0 | 15 |
| | 5.0 | 14 |
| | 10.0 | 16 |
| Crystalline bovine albumin | 0.5 | 16 |
| | 1.0 | 17 |
| | 5.0 | 13 |
| | 10.0 | 14 |
| *Experiment B* | | |
| None | | 4 |
| Bovine hemoglobin | 1.0 | 2 |
| | 5.0 | 16 |
| | 10.0 | 14 |
| Protamine sulfate | 0.5 | 0.5 |
| | 1.0 | 0.2 |
| | 10.0 | 0.1 |
| Fraction II bovine gamma globulin | 0.5 | 6 |
| | 1.0 | 7 |
| | 5.0 | 7 |

uptake. Figure 3 presents the rate curves for the three types of proteins. Of the proteins tested, none except the PPLO serum fraction (Difco) behaved as B-II. Bovine fraction III-$\beta$ globulin, crystalline bovine hemoglobin, and $\beta$-lactoglobulin behaved similarly to fraction V bovine albumin, although the first two mentioned did not allow nearly the total uptake permitted by fraction V. Bovine glycoprotein, fraction II bovine $\gamma$-globulin, and protamine gave results similar to crystalline bovine albumin. Figure 4 shows the rate curves for cholesterol uptake in the absence of protein and surface active agent and in the presence of these two components singly and together. Rapid adsorption in the presence of cholesterol alone occurs but does not reach the total uptake noted in the presence of proteins. This uptake can be accounted for by the effect of the large number of cells present. That the surface active agent alone has no direct effect on uptake is easily seen. The excess of cholesterol taken up in the absence of any added compound over that taken up in the presence of the surface active agent is due to precipitated sterol (Smith and Rothblat, 1960) and can be removed by washing the cells in a solution of lecithin. The presence of both protein and surface active agent slightly alters the rate curve and permits greater total uptake than in the presence of protein alone.

The specificity of the type of protein giving optimal growth and regulation of cholesterol uptake prompted an attempt to determine the

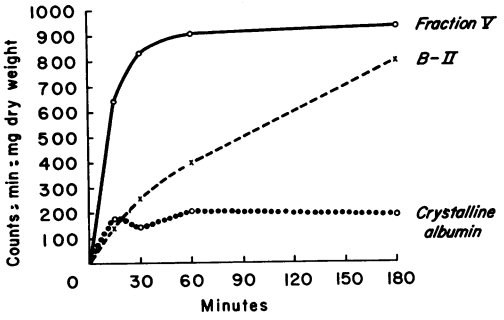


*Figure 3.* Patterns of uptake of cholesterol-4-$C^{14}$ in the presence of various proteins.

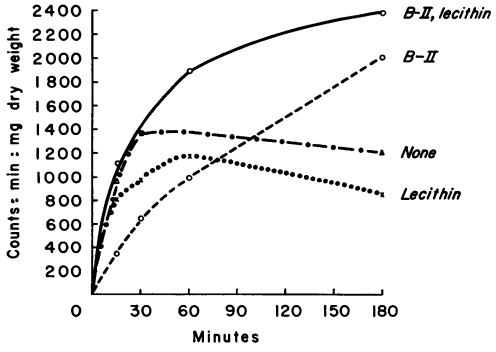


*Figure 4.* Effect of protein and surface active agent on the pattern of uptake of cholesterol-4-$C^{14}$.

nature of such specificity. The only approach attempted was that of protein end group blockage or destruction. It had been shown previously that enzymatic or chemical hydrolysis of the protein destroyed its growth promoting activity (Smith and Morton, 1951). Table 4 presents the data on growth response to B-II and fraction V bovine albumin when these proteins were treated as indicated. The only instance of complete growth suppression was treatment with heavy metals and *p*-chloromercuribenzoate. Although this result might at first glance indicate that sulfhydryl groups were required, it could not be the explanation since iodination destroys sulfhydryl groups but does not adversely effect growth to a significant degree. The probable reason is that the heavy metals and *p*-chloromercuribenzoate, not being tightly bound to the protein were released free in the growth medium and thus blocked the sulfhydryl groups of enzyme systems in the organisms. Cysteine could reverse this inhibitory activity. Combined blockage or destruction of sulfhydryl, amino and

TABLE 4

*Effect of end group destruction on ability of protein to support growth, strain O7*

| Reagent or Action | End Group | Growth Response | |
|---|---|---|---|
| | | B-II | Fraction V |
| 1-Fluoro-2,4-dinitrobenzene | $-NH_2$ | 0.31 | 2.12 |
| Na-2,4-dinitrobenzene sulfonate | $-NH_2$ | 1.00 | 1.25 |
| Acetylation | $-NH_2$ | 9.13 | 3.24 |
| Carbobenzoxy chloride | $-NH_2$ | 0.29 | 0.25 |
| Iodination | —SH (—OH) | 0.34 | 4.82 |
| *p*-Chloromercuribenzoate | —SH | 0 | 0 |
| Mono-iodoacetic acid | —SH | 0.007 | 0.75 |
| Mercuric chloride | —SH | 0 | 0 |
| *p*-Chloromercuribenzoate + cysteine (1.8 mg/ml) | | | 1.73 |
| Mercuric chloride + cysteine (1.8 mg/ml) | | 3.67 | 7.12 |
| Potassium periodate | —OH | 0.07 | 0.03 |
| Iodination + 1-fluoro-2,4-dinitrobenzene | —SH, (OH), $-NH_2$ | 1.50 | 0.65 |
| None | | 1.00 | 1.00 |

possibly hydroxyl groups had no effect on growth. Oxidation of the hydroxyl groups by periodate did significantly lower the growth response. Acetylation appeared to markedly stimulate growth.

The effect of periodate and 1-fluoro-2,4-dinitrobenzene treated B-II and fraction V albumin on the regulation of cholesterol uptake was examined. Periodate treatment did not alter the type of cholesterol uptake typically permitted by these two proteins, but it did decrease the total amount of cholesterol adsorbed by the cells. In the case of 1-fluoro-2,4-dinitrobenzene treatment some reduction in total uptake was also noted. However, an interesting effect was that the pattern of cholesterol uptake regulation of B-II was altered to one similar to that of fraction V (figure 5). The actual values depicted by the pairs of curves for B-II and fraction V are not comparable in this figure. The amount of total sterol taken up in the presence of B-II is

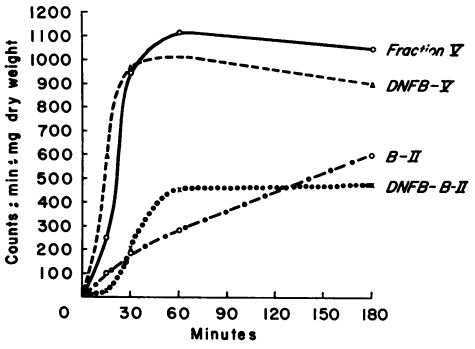


*Figure 5.* Comparison of the patterns of uptake of cholesterol-4-$C^{14}$ in the presence of 1-fluoro-2,4-dinitrobenzene labeled proteins and unlabeled proteins.

equal or greater than that taken up in the presence of fraction V after an exposure of 3 hr.

Thus it was concluded that although end groups on the protein might play some role, they are not the major requirement for a protein to regulate cholesterol uptake. These data indicate that regulation of cholesterol uptake appears to parallel the ability of a protein to support growth.

The function of the surface active agent was shown not to be regulation of cholesterol uptake (figure 4). The other possible functions include incorporation as part of the phospholipid of the cell proper, either intact or partially degraded, a source of oxidizable substrate, or effecting the aqueous solubilization of required sterol. The same type of analysis was used as with the protein, i.e., correlation of some activity with growth promotion.

Table 5 shows the growth response to various surface active agents. With the preparation of B-II used in this study, no absolute growth requirement for a surface active agent was noted, as was shown previously (Smith and Lynn, 1958). The concentration given in the table represents that level giving optimal growth. The growth stimulatory activity is not specific for any of the compounds tested. Previous results (Smith and Lynn, 1958) of a preliminary study indicated that intact lecithin was required. Oleate in a mixture simulating the components of lecithin was inactive. This was probably the result of too high a total concentration of toxic long-chained fatty acids.

That the stimulation of growth by long-chained fatty acids was due principally to their surface tension depressing action was further evidenced by the finding that in the presence of an optimal concentration of lecithin, no significant stimulation of growth occurred upon addition of levels of fatty acids (sodium oleate, linoleate, linolenate, arachidonate, stearate, palmitate, and myristate) varying from $10^{-7}$ to $10^{-4}$ final molarity. The highest concentration was found to inhibit growth.

Further substantiation of the assumption that the activity of these compounds was not that of a required nutrient was sought by assessing the amount of uptake by the cells. Table 6 shows that some oleate-1-$C^{14}$ was taken up by both resting and growing cells. This amount is fairly

TABLE 5

*Ability of surface active agents to stimulate growth of strain O7*

| Compound | Concn Giving Optimal Growth | Growth Response |
|---|---|---|
| None | 0 | 1.00 |
| Lecithin | $10^{-4}$ M | 1.96 |
| Tween 80 | $10^{-6}$ mg/ml | 1.60 |
| Na-oleate | $10^{-5}$ M | 3.72 |
| Na-linoleate | $10^{-5}$ M | 2.36 |
| Na-linolenate | $10^{-5}$ M | 1.46 |
| Na-arachidonate | $10^{-5}$ M | 1.83 |
| Na-myristate | $10^{-7}$ M | 1.23 |
| Na-palmitate | $10^{-5}$ M | 2.47 |
| Na-stearate | $10^{-5}$ M | 2.19 |
| Na-cholate | $10^{-6}$ M | 0.94 |
| Na-deoxycholate | $10^{-4}$ M | 1.09 |
| Na-lithocholate | $10^{-6}$ M | 1.56 |
| Na-taurocholate | $10^{-4}$ M | 0.84 |

TABLE 6

*Uptake of sodium oleate-1-$C^{14}$ by resting and growing cells*

| Strain | Nature of Cells | Uptake | |
|---|---|---|---|
| | | Amt | Total available oleate in cells |
| | | *μg/mg dry wt* | % |
| O7 | Resting | 0.07 | 2.0 |
| O7 | Growing | 0.06 | 0.1 |
| J | Resting | 0.27 | 4.0 |
| J | Growing | 0.16 | 0.2 |
| Laidlaw B | Resting | 0.10 | 0.8 |
| Laidlaw B | Growing | 0.03 | 0.1 |

TABLE 7

*Effect of surface active agents on aqueous solubility of cholesterol*

| Surface Active Agent | Concn | Soluble Cholesterol |
|---|---|---|
| | *mg/ml* | % |
| None | 0 | 7 |
| Lecithin | 0.0005 | 9 |
| | 0.001 | 17 |
| | 0.0025 | 30 |
| | 0.005 | 42 |
| | 0.01 | 65 |
| Na-oleate | 0.0005 | 48 |
| | 0.001 | 55 |
| | 0.0025 | 87 |
| | 0.01 | 87 |
| Na-cholate | 0.0005 | 8 |
| | 0.001 | 5 |
| | 0.01 | 9 |
| Tween 80 | 0.001 | 7 |
| | 0.0025 | 20 |
| | 0.005 | 26 |
| | 0.01 | 28 |
| Cephalin | 0.0005 | 10 |
| | 0.0025 | 23 |
| | 0.005 | 29 |
| | 0.01 | 32 |
| Na-arachidonate | 0.0005 | 12 |
| | 0.005 | 37 |
| | 0.01 | 39 |
| Na-linoleate | 0.0005 | 22 |
| | 0.001 | 26 |
| | 0.0015 | 47 |
| | 0.01 | 42 |
| Na-linolenate | 0.0005 | 13 |
| | 0.001 | 15 |
| | 0.005 | 19 |
| | 0.01 | 19 |
| Na-myristate | 0.0005 | 14 |
| | 0.01 | 15 |
| Na-taurocholate | 0.0025 | 14 |
| | 0.01 | 22 |
| Na-stearate | 0.001 | 40 |
| | 0.05 | 86 |
| Na-lithocholate | 0.001 | 21 |
| | 0.05 | 27 |
| Na-palmitate | 0.0025 | 33 |
| | 0.01 | 34 |
| | 0.05 | 91 |

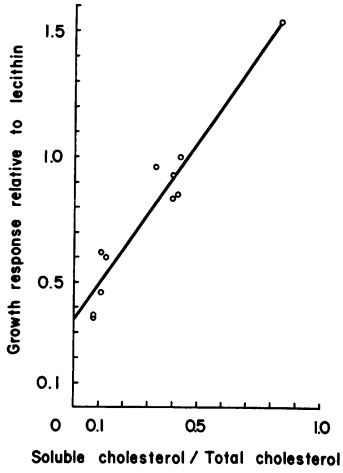


*Figure 6.* Correlation of growth stimulation and aqueous solubilization of cholesterol by surface active agents.

constant when measured as micrograms per mg dry weight in the presence of variable amounts of available oleate and could not be removed by repeated washing of the cells. It is probable that this oleate was incorporated into the cells. However, it is unlikely that all growth stimulating surface active agents would be incorporated, because of the diversity of their structures.

Some attempt was made to determine whether lecithin as an example of a growth stimulating phospholipid, was degraded. Numerous experiments have indicated that no significant $O_2$ uptake occurs with sodium oleate as substrate. Incubation of lecithin with resting cells at pH 6.5 and 7.5 for 6 hr at 37 C resulted in no increase of titratible fatty acid. However, an increase in inorganic phosphate of about 0.2 to 0.6 μmole per 50 μmoles available lecithin occurred. This amount was considered insignificant as a basis

for considering that the sole function of lecithin lies in a degradation product.

The only significant activity of the surface active agents which correlated with their ability to stimulate growth was their capacity to increase the aqueous solubility of cholesterol. Table 7 presents the data on this activity.

Figure 6 is a plot of growth stimulatory activity of a given compound at its optimal concentration versus the per cent of 0.01 mg per ml cholesterol soluble at the same level. Although variation is inherent due to the mathematical manipulations involved in $C^{14}$ self-absorption correction and due to the method of growth quantitation a fair degree of correlation between the two properties exists.

## CONCLUSION

The data obtained in this study would indicate that the primary function of the protein growth requirement is regulation of uptake of cholesterol by the cells. A secondary but not sole function is one of detoxification of required surface active agents. The role of the surface active agents appears to be the aqueous solubilization of cholesterol, thereby making it more available to the cells. Among the other possible functions, none appear to correlate with the ability of a compound to support growth.

One important question not answered by this study is the manner by which a protein regulates cholesterol uptake. It is extremely unlikely that the protein and cholesterol together with a phospholipid or surface active agent form a lipoprotein in vitro. On the other hand an intact lipoprotein could function as a source of cholesterol and its uptake regulation mechanism. End groups on proteins do not appear to be a specific requirement. That the protein possesses a charge on its molecule does appear essential since the orderly process of sterol uptake becomes disorganized near or at the isoelectric point of the protein and cells (Smith and Rothblat, 1960). The rapid irreversible adsorption of soluble cholesterol in the absence of protein indicates that uptake can occur without the protein being present. Likewise similar adsorption occurs when the protein present is at its isoelectric point. However, when the protein possesses a charge, regulation of uptake occurs. Thus the protein may in some manner be effecting a loose linkage between it and the sterol gradually surrendering to the appropriate cell sites.

One discrepancy appears in the data supporting a role of surface active agents in increasing the aqueous solubility of cholesterol. If this requirement of a surface active agent is a passive one, none should be taken up by the cells. The uptake of sodium oleate may not be significant to this function since a saprophytic strain, Laidlaw B, not requiring sterol, also takes up oleate. Between the two parasitic strains examined, the amount of oleate taken up differs. A similar difference, although possibly only coincidental, has been noted in the amount of nonvolatile fatty acids present in cholesteryl esters of these two strains (Lynn and Smith, 1960). The fate of the oleate taken up by the cells is unknown but it probably is incorporated as a part of the cholesteryl ester and phospholipid components of the cell.

## SUMMARY

The ability of a protein to support growth of pleuropneumonia-like organisms appears to correlate with its ability to regulate the uptake of cholesterol by the cells. Proteins capable of supporting growth are all capable of neutralizing the lytic activity of surface active agents. Some proteins inactive for growth also possess this latter activity. No protein is taken up by the cells nor do the cells degrade it. The surface active agent required for growth functions by increasing the aqueous solubility of cholesterol. Some incorporation by the cells of sodium oleate was noted.

## REFERENCES

BERGMANN, M., AND L. ZERVAS 1932 Über ein allegemeines Verfahren der Peptid-Synthese. Ber. deut. chem. Ges., **65**, 1192–1201.

CHINARD, F. P. AND L. HELLERMAN 1954 Determination of sulfhydryl groups in certain biological substances. Methods of Biochem. Anal., **1**, 1–26.

DESNUELLE, P. 1953 The general chemistry of amino acids and peptides. In *The proteins*, vol. I, pp. 87–180. Edited by H. Neurath and K. Bailey. Academic Press, Inc., New York.

DU VIGNEAUD, V., AND C. E. MEYER 1932 The racemization of amino acids in aqueous solution by acetic anhydride. J. Biol. Chem., **98**, 295–308.

EDWARDS, D. G., FF., AND W. A. FITZGERALD

1951 Cholesterol in the growth of organisms of the pleuropneumonia group. J. Gen. Microbiol., **5,** 576–586.

EISEN, H. N., S. BELMAN, AND M. E. CARSTEN 1953 The reaction of 2,4-dinitrobenzenesulfonic acid with free amino groups of proteins. J. Am. Chem. Soc., **75,** 4583–4584.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., **66,** 375–400.

FRAENKEL-CONRAT, H., J. I. HARRIS, AND A. L. LEVY 1955 Recent developments in techniques for terminal and sequence studies in peptides and proteins. Methods of Biochem. Anal., **2,** 359–426.

GOLDSTEIN, N. P., J. H. EPSTEIN, AND J. H. ROE 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.

LYNN, R. J., AND P. F. SMITH 1960 Chemical composition of PPLO. Proc. N. Y. Acad. Sci., **79,** 493–498.

MORTON, H. E., P. F. SMITH, AND P. R. LEBERMAN 1951 Investigation of the cultivation of pleuropneumonia-like organisms from man. Am. J. Syphilis, Gonorrhea, Venereal Diseases, **35,** 361–369.

PRESSMAN, D., AND H. N. EISEN 1950 The zone of localization of antibodies. V. An attempt to saturate antibody-binding sites in mouse kidney. J. Immunol., **64,** 273–279.

RODWELL, A. W. 1956 The role of serum in the nutrition of *Asterococcus mycoides*. Australian J. Biol. Sci., **9,** 105–116.

SMITH, P. F. 1955 Amino acid metabolism by pleuropneumonia-like organisms. I. General catabolism. J. Bacteriol., **70,** 552–556.

SMITH, P. F. 1960 Nutritional requirements of PPLO and their relation to metabolic function. Proc. N. Y. Acad. Sci., **79,** 508–520.

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

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

SMITH, P. F., AND G. H. ROTHBLAT 1960 Incorporation of cholesterol by pleuropneumonia-like organisms. J. Bacteriol., **80,** 842–850.

SMITH, P. F., AND S. SASAKI 1958 Stability of pleuropneumonia-like organisms to some physical factors. Applied Microbiol., **6,** 184–189.

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

STADTMAN, E. R., G. D. NOVELLI, AND F. LIPMANN 1951 Coenzyme A function and acetyl transfer by the phosphotransductase system. J. Biol. Chem., **191,** 365–376.