A LIPOPROTEIN AS A GROWTH FACTOR FOR CERTAIN PLEUROPNEUMONIALIKE ORGANISMS[1]

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Received for publication May 28, 1954

Certain strains of pleuropneumonialike organisms fail to multiply *in vitro* in the absence of factors found principally in mammalian sera. Results of attempts to isolate and characterize this requirement have led to two conclusions. Smith and Morton (1951, 1952) found that a heat stable protein permitted growth to occur when added to a complex basal medium. Edward and Fitzgerald (1951) and Edward (1953) successfully cultivated certain strains on a complex basal medium to which were added cholesterol, bovine serum albumin, and an acetone insoluble lipid fraction derived from egg yolk. Additional study was therefore required to elucidate these seemingly divergent results. Consequently, the protein described by Smith and Morton has been studied chemically and biologically with the eventual finding that it is a lipoprotein similar to or a degradation product of *alpha*-1 lipoprotein found in mammalian sera.

MATERIALS AND METHODS

The methods for assaying the growth promoting activity of various compounds were the same as described previously (Smith and Morton, 1951). The basal medium unless otherwise indicated was bacto-PPLO agar. In some cases where only small amounts of fractions were available, thallium acetate, 1:1,500 (Morton and Lecce, 1953), was added to prevent growth of contaminants in place of sterilization by filtration through Selas 02 filters. Sterols and other water insoluble compounds were added to the basal medium as methanol solutions. Two strains of pleuropneumonialike organisms obtained from humans served as test strains. One strain, Campo L, was very easily cultivated while strain 48 was very fastidious in its growth. All of the stock strains of human origin were tested on the final optimal medium.

Free and total cholesterol were determined by a modified method of Schoenheimer and Sperry (Hawk *et al.*, 1947), phospholipid by the method of Youngburg (Hawk *et al.*, 1947), and protein by the turbidimetric method of Stadtman *et al.* (1951). Methyl orange and cholesterol binding by proteins was carried out according to the procedure of Klotz *et al.* (1946). Cholesterol in these binding experiments was determined spectrophotometrically at a wavelength of 230 mμ employing a Beckman DU spectrophotometer.

The source of the protein factor was bovine blood serum. The method for obtaining the purified protein, fraction B-II, was a modification of the method previously described (Smith and Morton, 1951, 1952). Whole bovine serum at a pH greater than 7.0 was diluted three to fourfold with distilled water. This dilute serum was heated for 20 minutes in a boiling water bath. Care must be taken that an even temperature be attained in the serum within two minutes. Upon cooling to room temperature, the pH was lowered to 6.0 with N hydrochloric acid and the precipitated proteins removed by filtration or centrifugation. The filtrate was reduced in volume by evaporation in cellophane casings on which was directed a stream of air. When the volume was reduced about tenfold, the pH was adjusted to about 7.0–7.2 with N sodium hydroxide and the solution dialyzed for 72 hours against distilled water. If the preparation contained more than one electrophoretic component, repeated precipitation of inert protein at pH 4.8 together with dialysis of the preparation prior to readjustment of the pH to 7.0 was carried out. Any precipitate which formed was removed by centrifugation. The final solution was water clear. Further concentration was achieved by freeze-drying. Storage of the preparation in the refrigerator or in a freezer may lead to the precipitation of some inactive protein.

[1] This work was supported in part by a contract between the University of Pennsylvania and the Office of Naval Research.

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This was subsequently removed by centrifugation. This modification permitted the preparation of large amounts of the pure component sufficient for analytical studies. The final product was shown to consist of only one component by means of paper electrophoresis at various pH's ranging from 4.0 to 10.0 and to contain only a trace of impurity of slightly faster sedimentation by ultracentrifugation. A level of 0.5 mg per ml of the final medium permits optimal growth.

Paper electrophoresis was carried out in the conventional manner at 6 C in various phosphate, veronal, and borate buffers of ionic strength 0.1 employing a current of 15 ma. The apparatus used was manufactured by Bender and Hobein, Munchen 15, Lindwurmstrasse 71, Germany. The paper strips were stained with bioblue (amidoblack 10-B) and decolorized with a methanol-glacial acetic acid mixture. Measurement of the electrophoretic patterns was performed with a photoelectric densitometer manufactured by Bender and Hobein.

Ultracentrifugation was carried out in a Model E Spinco analytical ultracentrifuge at 59,780 rpm employing a current of 92 v.[3] The protein to be analyzed consisted of 1.5 per cent solution in 0.15 M sodium chloride plus 0.02 M monopotassium phosphate at pH 5.2. Duration of the run was two hours.

Molecular weight determination was also performed by osmotic pressure measurement according to the method of Mallette (1954). A 0.5 per cent solution of fraction B-II in 0.1 M acetate buffer containing 0.5 M sodium chloride was used to determine osmotic pressure. Determinations were made at pH 5.2 and 6.0. Control determinations were made with bovine serum albumin at pH 5.2.

Extraction of proteins to remove lipids was accomplished by treating the dry protein with large volumes of methanol at room temperature. Methanol extraction of the basal medium failed to remove all of the active lipids or removed required nonlipoidal components. Therefore the basal medium in the form of broth was extracted twice at pH 4 with two volumes of ether, followed by readjustment of the pH to 7.8 and addition of 1.5 per cent agar.

Other proteins used in this study, bovine serum albumin and β-lactoglobulin from milk, were obtained from Armour and Co. The sterols, phospholipids, and fatty acids were obtained from commercial sources.

[3] Ultracentrifugal analysis was kindly performed by Dr. S. Soroff, the Institute for Cancer Research and the Lankenau Hospital Research Institute, Dr. Stanley P. Reimann, Director, Fox Chase, Philadelphia 11, Pa.

EXPERIMENTAL RESULTS

There were two objectives to this study. One constituted the identification of the protein growth factor, the other the determination of all of the required factors supplied to the basal medium by the protein supplement. The first objective was satisfied by an analysis of the properties and constituents of the purified protein preparation, fraction B-II, and the second by carefully regulated addition of various components found in the protein fraction to the culture medium.

Chemical analysis of the growth factor. Previous publications (Smith and Morton, 1951, 1952) described fraction B-II as a heat stable, relatively low molecular weight protein. When added to the basal medium the test strains grew, but this growth was not equivalent to the optimum. No cholesterol was detectable in fraction B-II. As will be subsequently shown this was due to the fact that all of the cholesterol was in the bound form. The original work by Smith and Morton (1951) also pointed out that serum proteins extracted in the dry state with fat solvents did not reduce their activity. This finding seemed to indicate that lipoprotein might be excluded. However, it was subsequently determined that the crude basal medium contained enough essential lipoidal substances to permit growth to occur, thus masking the total effect and probable identity of the protein factor. Employing a basal medium extracted to remove lipids, it was found that the purified lipid-free protein was incapable of supplying the necessary requirements. It was further suspected that the intact growth factor was of lipoproteinaceous character since the ether extracted protein permitted growth while the methanol extracted protein was deficient. On this suspicion various analyses were made on the purified intact protein. Table 1 lists these properties in comparison with the properties of the known lipoproteins of serum.

Demonstration of the one component of fraction B-II by paper electrophoresis is shown in

TABLE 1

Properties and constitution of fraction B-II, alpha-1 lipoprotein, and beta-1 lipoprotein

Characteristic	B-II	*alpha*-1 Lipoprotein‡	*beta*-1 Lipoprotein§
Per cent of plasma protein	3	3	5
Per cent free cholesterol	trace	none	8.3
Per cent bound cholesterol	7.6	present	39.1
Per cent phospholipid	0.07	present	29.3
Electrophoretic mobility*	7.98×10 μ/sec/v/cm (similar to *alpha*-1 globulin)	similar to *alpha*-1 globulin	similar to *beta*-1 globulin
Isoelectric point†	5.2	5.2	5.4
Sed. constant S_{20}	3.75	5	7
Molecular weight	1.35×10^5‖	2×10^5	1.3×10^6

* Electrophoretic mobility, employing an Aminco-Stern apparatus (American Instrument Co., Catalog no. 5-8000), was performed at 2.7 C in pH 8.6 veronal buffer of ionic strength 0.1 at a current of 15 ma.

† Isoelectric point determined by immobility in electric field employing paper electrophoresis with phosphate buffers at ionic strength 0.1 and a current of 15 ma.

‡ Data of Cohn *et al.* (1950).

§ Data of Oncley (1953).

‖ Molecular weight by osmotic pressure measurement.

figure 1. The density of the stained protein is plotted against the length of the area of the electrophoretic pattern. Addition of this fraction to fraction A which exhibits three distinct electrophoretic components, resulted in the increase of only the central component of the electrophoretic pattern of fraction A (figure 2). Figure 3 shows the sedimentation pattern of fraction B-II.

The molecular weight of fraction B-II was found to be 1.35×10^5 by osmotic pressure measurement. In contrast to this molecular weight is that one determined from the sedimentation data. The latter method gave a molecular weight of 3.9×10^4 when the protein molecule was assumed to be spherical in shape, nonhydrated, and to have a partial specific volume of 0.749. The molecular weight determined by osmometry is assumed to be a more accurate approximation since lipoproteins display a marked sedimentation dependence on protein concentration. A more detailed ultracentrifugal analysis of the protein would be necessary to arrive at an accurate molecular weight by sedimentation velocity. Furthermore, lipoproteins are not usually spherical in shape (Oncley, 1953).

The unavailability of any known lipoproteins of serum prevented the actual comparison of the three proteins. The data, summarized in table 1, demonstrate a possible relationship between fraction B-II and *alpha*-1 lipoprotein. The lower

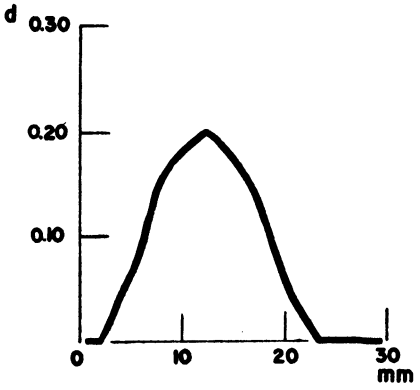

Figure 1. Electrophoretic pattern of fraction B-II after 24 hours in pH 8.6 veronal buffer at ionic strength 0.1 employing a current of 15 ma.

molecular weight of B-II could be accounted for by loss of phospholipid during the rather drastic method of fractionation. Nevertheless in regard to growth promoting ability of fraction B-II, this paucity of phospholipid is alleviated by the presence of such compounds in the crude basal medium.

Biological analysis of the growth factor. Methanol extraction of fraction B-II separated the growth factor into two essentially inactive fractions. The lipid-free protein, however, when added to the unextracted basal medium did support subopti-

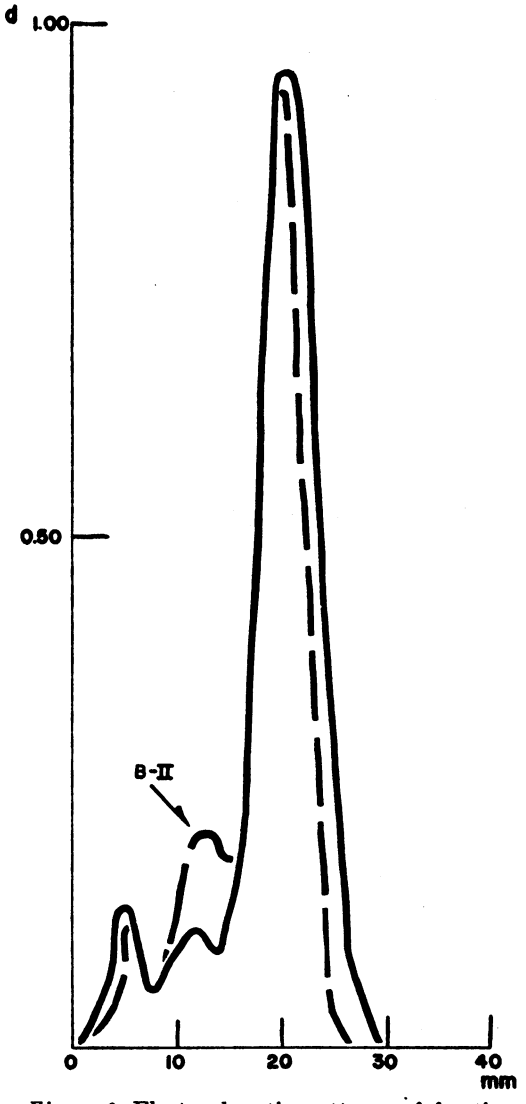

Figure 2. Electrophoretic patterns of fraction A (solid line) and fraction A + B-II (broken line).

mal growth. The addition of both the lipid-free protein and the methanol extract to the extracted basal medium was required to give optimal growth equivalent to that obtained when the intact protein is added to the intact basal medium. Illumination of the concentrated methanol extract with ultraviolet light showed it to possess some luminescence. Spectrophotometric examination revealed a density peak at 230 mμ similar to cholesterol. Substitution of the methanol extract with varying levels of cholesterol permitted optimal growth in the intact but suboptimal growth in the extracted basal medium. No toxicity was noted when cholesterol was added in amounts varying from 0.005 to 0.50 mg per ml.

The absence of free cholesterol in the protein indicated that cholesterol in itself was not the actual compound required. Since the presence of bound cholesterol implies the existence of cholesteryl esters, a number of these compounds were used to replace cholesterol. Cholesteryl laurate at a level of 0.01 mg per ml could substitute for the unesterified sterol. Acetate, palmitate, stearate, and oleate esters of cholesterol possessed very poor or no activity. That these latter esters are inactive is in accord with the findings of Edward and Fitzgerald (1951).

The substitution of cholesteryl laurate for the methanol extracted basal medium did not completely restore the original activity of the final medium. The other major component of lipoproteins is phospholipid. Edward and Fitzgerald (1951) have shown that an acetone insoluble lipid derived from egg yolk was stimulatory for the growth of pleuropneumonialike organisms. The extracted basal medium was supplemented with purified preparations of either cephalin or lecithin of animal origin in addition to cholesteryl laurate and the lipid-free protein. The combination of 0.5 mg per ml lipid-free protein, 0.01 mg per ml cholesteryl laurate, and 0.005 mg per ml of lecithin permitted optimal growth to occur. Cephalin was much less active when added together with the cholesteryl laurate but proved more active with cholesterol than did the lecithin. Replacement of cholesteryl laurate with cholesterol and sodium laurate on an equimolar basis did not support growth equivalent to that given with the intact ester. These data are summarized in table 2. The amount of phospholipid added was critical. A level of lecithin greater than 0.005 mg per ml resulted in some growth inhibition. The levels of lipoidal components which gave optimal growth were nearly equivalent to those levels found in the crude final medium.

It was of interest to determine the specificity of the protein moiety of the lipoprotein for the growth of pleuropneumonialike organisms as well as the possibility of its function as a neutralizer of fatty acid toxicity. The dose responses to three proteins, namely fraction B-II, bovine serum albumin, and β-lactoglobulin from milk, were studied. All three proteins were rendered lipid-free and supplemented with the necessary components. It was found that optimal growth

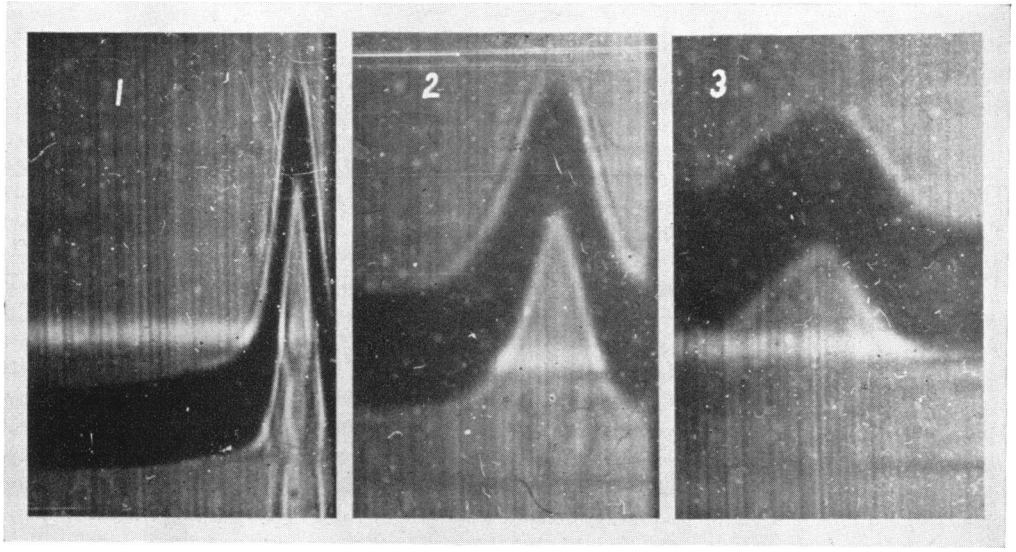

Figure 3. Sedimentation pattern of fraction B-II in 0.02 M pH 5.2 phosphate buffer and 0.15 M sodium chloride. Photographs were taken at 40 minute intervals.

TABLE 2

Growth promoting properties of various combinations of components of fraction B-II

Combination of Components	Relative Growth†	
	Strain Campo L	Strain 48
Protein* + cholesterol	3	2
Protein + cholesteryl laurate	3	2
Protein + cholesterol + lecithin	4	3
Protein + cholesteryl laurate + lecithin	4	4
Protein + cholesterol + Na laurate + lecithin	4	3

* The protein and the basal medium in all cases were lipid-free. Protein was added at a level of 0.5 mg per ml; cholesterol and cholesteryl laurate at levels of 0.01 mg per ml; lecithin at a level of 0.005 mg per ml; Na laurate at a level of 0.006 mg per ml.

† 4 = optimal growth; 3 and 2 = progressively poorer growth.

occurred in the presence of 0.5 mg per ml of fraction B-II, while 5.0 mg per ml of the other two proteins were required to achieve the same result.

The substitution of the intact lipoprotein with the lipid-free protein together with varying levels of sodium oleate or "tween 80" in the intact basal medium presented some interesting results. The fastidious strain, 48, failed to grow. On the other hand, strain Campo L developed fair growth. Furthermore the protein could be replaced with one per cent starch or charcoal (Norit) without any diminution in activity. Although growth with these substances never equalled that obtained under optimal conditions, it is of concern because of the alteration in other properties of the Campo L strain during several years cultivation on artificial medium. Another example of this change is the development of frank turbidity in liquid culture.

A measure of the ability of proteins to neutralize fatty acids is their capacity to bind anions, an example of which is methyl orange. Following the procedure of Klotz *et al.* (1946) results of methyl orange binding for bovine serum albumin and β-lactoglobulin were the same as those reported in the literature (Klotz and Urquhart, 1949). However, fraction B-II bound only a trace or no methyl orange under the same conditions. When cholesterol was substituted for methyl orange, bovine serum albumin was found to bind 8.6 moles cholesterol per mole of protein (assuming a molecular weight of 6.8×10^4); β-lactoglobulin, 7.7 moles per mole protein (assuming a molecular weight of 3.5×10^4); fraction B-II, 33.5 moles per mole of protein (molecular weight of 1.35×10^5). Some correlation exists with the ability of proteins to bind cholesterol and to pro-

mote growth of pleuropneumonialike organisms. Proteins completely inactive in regard to growth promotion were found to possess even less binding capacity for cholesterol than bovine albumin.

DISCUSSION

The results of this study tend to elucidate the seemingly divergent conclusions reached by Smith and Morton (1951, 1952) and by Edward and Fitzgerald (1951) and Edward (1953). While the latter investigators found that bovine albumin, cholesterol, and an acetone insoluble lipid supported optimal growth of most strains, our results indicate that the actual growth factor supplied by serum is a lipoprotein containing components similar in nature to those mentioned above. Edward and Fitzgerald (1951) found that the acetate, stearate, phthalate, and oleate esters of cholesterol were inactive alone or in the presence of starch, bovine albumin, or acetone insoluble lipid fraction of egg yolk. This is in accord with our findings when using these esters with lipid-free protein and lecithin. However, cholesteryl laurate, a compound not tried by Edward and Fitzgerald, possessed the required activity. Probably a mixture of esters, which is undoubtedly the composition of the natural material, would possess even more activity. These investigators further reported that purified preparations of lecithin or cephalin failed to replace their acetone insoluble lipid fraction. The level added to the medium is a critical factor. Concentrations of lecithin of animal origin less than or greater than 0.005 mg per ml fail to give optimal growth. Although the purity of the lecithin employed in our experiments was only 90 per cent, the very small amounts employed make it unlikely that an impurity is the active material.

A few complicating factors exist in the elucidation of the growth requirements of pleuropneumonialike organisms. The lack of suitable methods for the quantitation of growth hampers critical experimentation. None of the present methods used to assay microbial growth is feasible for quantitating growth of pleuropneumonialike organisms on a scale extensive enough for experiments involving study of growth requirements.

It has been the suspicion among workers in the field of pleuropneumonialike organisms that this group of microorganisms possesses great heterogeneity. Recently as a result of the increased pace of research in this field, soundly based speculations on the part of two investigators have given rise to the grouping of pleuropneumonialike organisms into distinct classes. Nicol and Edward (1953) have described four different types based on their growth requirements, fermentative activity, and pathogenicity for mice. Freundt (1953) classed the strains he studied into three types. The majority of the strains isolated from the human genital tract require serum for growth and possess very weak metabolic activity in regard to most tested substrates. Two strains were found by Freundt to be indistinguishable from the bovine pleuropneumonia organism. It is becoming more apparent that study of a few select strains fails to elucidate the properties of all pleuropneumonialike organisms. Such may eventually be the case with the lipoprotein factor.

The growth requirement of certain pleuropneumonialike organisms for a lipoprotein raises the question of its function. Fatty acid detoxification does not appear to be its principal action. The fact that lipoproteins in the mammalian blood appear to function as carriers of water insoluble compounds tends to suggest a similar explanation for the function of this lipoprotein in microbial growth. Poetschke (1953, *personal communication*) has also suggested this explanation in regard to work on L-forms of corynebacteria. This investigator has actually shown that when cholesterol is added to the medium inoculated with L forms, its disappearance from the medium is followed by the appearance of crystals of cholesteryl esters in the L forms. A study of the function of the lipoprotein in the growth of pleuropneumonialike organisms is now in progress.

SUMMARY

The protein growth factor for pleuropneumonialike organisms present in mammalian blood sera has been identified as a lipoprotein containing only bound cholesterol and phospholipid. Its properties possess a striking similarity to those of *alpha*-1 lipoprotein. Employing a lipid-free basal medium, this lipoprotein factor can be replaced to give optimal growth with the lipid-free protein, cholesteryl laurate, and lecithin of animal origin. The protein moiety is not greatly specific since larger amounts of bovine serum albumin and β-lactoglobulin from milk will also support optimal growth.

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