STUDY OF THE EFFECT OF PROTEIN COMPONENTS IN BLOOD SERUM UPON THE GROWTH OF BRUCELLA ABORTUS

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THESIS

STUDY OF THE EFFECT OF PROTEIN COMPONENTS IN BLOOD SERUM UPON THE GROWTH OF *BRUCELLA ABORTUS*

By

Betty Virginia Baltzer

AN ABSTRACT

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ABSTRACT

Inhibition, both of colonial development on tryptose agar and of multiplication in tryptose broth, when seeded with cells of the fastidious, CO_2-dependent Brucella abortus Type II (Wilson), hereafter referred to as strain 119, was shown to be corrected by the addition of whole bovine serum to either medium. This response was assayed by the filter paper disc method on agar, and by the resultant degree of turbidity in broth.

The plasma protein fraction responsible for effecting growth was found to be albumin. Neither bovine plasma fraction II (gamma globulin), nor fraction III-1 (beta globulin), effected any growth response.

Attempts were made to improve the growth-enhancing action of albumin both by heat and chemical denaturation, and by digestion with three proteolytic enzymes -- pepsin, trypsin, and papain. However, each of these procedures depressed albumin activity. A milder heat treatment, performed while protecting albumin with saturated sugar solutions, did not alter the growth-stimulatory capacity of albumin.

Beside trial of the plasma protein fractions, incorporation in tryptose agar of two synthetic agents, Tween 80 and Span 80, showed that only the former, water-miscible agent enhanced growth, and the response was nearly equal to that obtained from the addition of bovine plasma albumin.

Incorporation in tryptose agar of the sodium salts of two fatty acids gave the following results: lactic acid inhibited strain 119 at

1.07×10^{-1} M concentration, but did not inhibit the typical, aerobic Br. abortus strain 2308; oleic acid inhibited strain 119 at 3.28×10^{-6} M concentration, and also inhibited strain 2308 at 6.6×10^{-3} M, a higher concentration.

The technique of incorporating inhibiting agent (oleic acid) in tryptose agar and of adding enhancing agent (albumin or Tween 80) to the paper disc, revealed that these latter agents reversed the toxicity of fatty acid. That is, the diameter of the growth zone around the paper disc widened as weaker concentrations of inhibitory agent were mixed in the agar. Furthermore, growth-stimulation, better even than that obtained from enhancing agent alone, resulted from interacting the threshold inhibitory concentration of oleic acid (in agar) with either albumin or Tween 80 (on paper disc).

The growth-enhancing action of albumin (as well as Tween 80) was attributed, not to a nutritive effect, but rather to a protective, detoxifying action, since the binding of ions by this protein, especially fatty acid anions, notably surpassed that of any other protein so examined (1).

The protective action of albumin was established by treating tryptose broth with an adsorbing agent known to lack nutritive value, Norite-A. Whether Norite-A was removed before inoculation, or was present throughout the growth period, Norite-A-treated broth subsequently supported as ready multiplication of a minimum number of bacterial cells of strain 119 as did tryptose broth containing 0.005 percent albumin. Thus, prior to treatment with either binding agent, the tryptose constituent used to prepare broth and agar apparently contained some agent free to inhibit growth of strain 119. This inhibitor was likened to a fatty acid.

Apparently some strains of Brucella are far more sensitive to fatty acids than others. Yet, if firmly bound or detoxified, the amount of fatty acid present in the medium was of no consequence. Thus, the concentration as well as the time of adding the binding agent determined whether the action of the inhibitor would be bactericidal, bacteriostatic, without effect, or perhaps even beneficial.

REFERENCE

1. Klotz, I. M. (1949). The nature of some ion-protein complexes. Cold Spring Harbor Symposia On Quantitative Biology, Long Island Biological Association, Cold Spring Harbor, N. Y., 14, 97- 112.

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INTRODUCTION

One of the most challenging problems that continues to confront study of all microbial life, has been that of promoting growth artificially, extracellularly. The more closely allied the life cycle of the microorganism has been to intracellular development, the more difficult has been the solution of this problem. Yet, again and again reports have come of the successful growth of fastidious bacteria following the incorporation of blood serum or plasma in culture mediums.

Though not obligate intracellular parasites, members of the genus *Brucella* frequently resist primary isolation, and some strains, more fastidious than others, characteristically do not grow well on most culture mediums. The result of employing blood serum in a culture medium for the growth of one such fastidious *Brucella abortus*, and the serum protein component responsible for growth is reported here, as well as the probable explanation of the role of action of this component.

GENERAL PROCEDURES

A. MATERIALS

Cultures. An S-type strain of an atypical, CO_2-dependent Br. abortus, 119, classified as Type II (Wilson)(1) and known to be fastidious, was used in all investigative studies. Two other S-type strains were used for comparative purposes: a typical, CO_2-dependent Br. abortus, 3038, and a non-CO_2-dependent Br. abortus, 2308.

Agar medium. One basic agar medium, tryptose, was used throughout the investigation. It was prepared in the laboratory from the constituents recommended by the manufacturer. One lot of tryptose was used in all preparations. Since an alkalinity higher than that of the unadjusted medium was always desired, the final pH was controlled by a predetermined quantity of 5 percent Na_2CO_3, added before autoclaving. Unless indicated, all mediums prepared for the CO_2-dependent strains were adjusted to obtain a final pH of 7.5; mediums for the aerobic strain were adjusted to pH 7.1 - 7.3. These initial pH values were determined as optimum in a previous study by Huddleson (2).

Tryptose agar fortified with killed brucella cells and variously used either experimentally or as a control type medium, was prepared by the addition before autoclaving of heat-killed cells of Br. abortus 2308 to a final concentration of 2×10^9 cells per ml. of agar.

Before using, all agar plates were dried 48 hours at 37°C.

Liquid mediums. Tryptose and trypticase soy mediums were prepared in the laboratory as follows:

Tryptose broth medium:
Bacto-tryptose .....2.0 gms.
Dextrose .....0.1 gms.
Sodium chloride .....0.5 gms.
Distilled water .....100 mls.
Dissolve ingredients at room temperature by stirring.
Adjust pH to 6.8 with .1 N HCl.

Trypticase soy broth medium:
Trypticase .....1.7 gms.
Phytone .....0.3 gms.
Sodium chloride .....0.5 gms.
Dextrose .....0.25gms.
Distilled water .....100 mls.
Dissolve ingredients at room temperature by stirring.
pH not adjusted.

Both mediums were dispensed in 20 ml. amounts into cotton plugged 50 ml. serum bottles, and autoclaved at 120°C. for twenty minutes.

B. METHODS

Inoculation of agar mediums. Br. abortus cultures were maintained on slants of Albimi brucella agar. Slant cultures of 119 and 3038 were incubated 36 hours under 5 percent CO_2. Slant cultures of 2308 were incubated 24 hours aerobically. Cells were suspended in tryptose diluting fluid (0.5 percent NaCl and 0.05 percent tryptose) to a concentration of 2×10^9 total cells per ml. as measured by the Libby Photronreflectometer.

When agar plates were inoculated with either of the CO_2-dependent strains, a 1:375,000 dilution of the cell suspension was made. One ml. of this dilution, containing from 2,500 to 3,000 viable cells, was flooded over the surface of the agar and the excess liquid poured off within two

minutes. Inoculation with Br. abortus 2308 was done similarly except that the one ml. inoculum was made to contain approximately 2,000 viable cells. Since plate count reproducibility was not the objective, this procedure proved easy, and a successful one for the even distribution of approximately 1,200 isolated bacterial cells. The inoculated plates were air dried upon a level surface in the incubator for at least two hours following inoculation.

The filter-paper disc method, first described by Vincent and Vincent (3), was used to assay the effect of an agent upon colonial development. A sterile disc was placed in the center of an inoculated plate, followed in five to ten seconds by the addition of 0.1 ml. of a liquid suspension of the agent. The plates were then air dried for an hour on a level surface in the incubator. This ensured uniform diffusion of the material into the agar medium.

Plates inoculated with the CO_2-dependent Br. abortus strains were incubated, inverted, for 72 hours in a 5 percent CO_2 atmosphere and partial vacuum, in glass jars or in dessicators, in which was also a beaker containing five grams of Dehydrite crystals. The latter prevented excessive condensation of moisture within the plates. Plates inoculated with strain 2308 were incubated aerobically for 72 hours.

Measurement of colonial development. At the end of this time a dissecting-type, binocular stage microscope (x 12) with a micrometer eyepiece was employed to measure the diameters of colonies present on each plate, and changes in the growth of colonies around the filter paper disc to which an agent had been added.

On the plates without filter paper discs, the colonial diameter appearing most often was recorded. Also, a plate colony count was made.

When colonial growth was enhanced by addition of agent to the filter paper disc, the diameters of the colonies were of maximum size nearest the paper disc and became progressively smaller as the amount of the agent decreased by diffusion through the agar. The diameters of the two size extremes were recorded. Also measured was the radius from the disc over which the enhanced growth extended. If, however, the added agent caused inhibition, the radius from the disc of depressed colonial development was likewise recorded. In the latter case, the colonies bordering the zone of inhibition were smaller than those farther away.

Inoculation and measurement of growth in liquid mediums. The essence of the procedure followed here has been described by Huddleson (4). Because evaluation of the growth promoting properties of broth mediums is more decisive when inoculated with a minimum number of bacterial cells, the final dilution of the 2 x 10^9 cell suspension (described previously) was such that twenty to twenty-five cells of strain 119, or ten to fifteen cells of strain 3038 (determined from agar plate colony count) were placed in each bottle of broth. Next, the agent (agents) to be tested for growth-promoting effect was added in a suitable concentration so that dilution of the medium was not appreciable. Last, the substance added to all bottles, was 0.5 ml. of a freshly prepared, aqueous, 8 percent $NaHCO_3$ solution, sterilized by filtering through a D-8 Hormann pad in a Seitz filter. This served to adjust the initial pH, as well as to maintain a favorable pH during incubation by controlling the quantity of CO_2 within the bottle. Within the ensuing two minutes after addition of the $NaHCO_3$, the cotton plugs were replaced with rubber diaphragm stoppers, and the bottles then incubated for 72 hours at 37°C. Surveillance was

kept for the initial appearance of turbidity indicating growth, and the increase in visible turbidity was recorded at 48 and at 72 hours. As a check, the pH level was determined by glass electrode immediately after unstoppering each bottle at completion of incubation.

EXPERIMENTS AND RESULTS

1. The effect of whole bovine serum on colonial growth. During the course of previous studies by Huddleson (2), from which stems the report that presence of brucella cells in mediums accelerate the reproduction rate of Brucella abortus, especially when small inoculums are used, another agent, aged blood serum, was noted to have a similar influence on this species. Therefore, to make a more thorough study of this latter agent, various saline dilutions of sterile whole bovine serum (Armour and Company) were assayed for their growth-promoting effect on the colonial development of the atypical strain 119. This was done by the filter paper disc method already described (plate 1). The results (table 1) show that the concentration of whole serum influences the maximum size attained by colonies, and the distance that colonial development extends from the disc. The latter factor affects the colony count around the disc.

2. The effect of blood plasma proteins on colonial growth. In order to determine which of the protein constituents of bovine blood plasma was responsible for the growth-promoting effect of whole serum on the Br. abortus under study, the following experiment was designed to assay the effect of beta and gamma globulin, and crystalline bovine plasma albumin, as well as whole bovine serum. The filter paper disc method was used. These fractions (Armour's) were easily reconstituted in physiological saline, to a concentration per ml. near that found in whole bovine serum. No pH adjustment was made on these solutions. Alpha globulin,

TABLE 1

THE EFFECT OF CONCENTRATIONS OF WHOLE BOVINE SERUM ON COLONIAL GROWTH
(Paper disc method)

Concentration of Protein (percent)	Radius of Growth from Disc[a]	Colony Diameter[a]	Colony Count Around Disc
8.0	15	1.3 - 0.1	310
4.0	12	1.2 - 0.1	170
0.4	8	1.0 - 0.1	180
0.16	5	0.4 - 0.1	90
0.08	2	0.2 - 0.1	10
0.008	0		0

[a]In mm.

designated Fraction IV (Armour's), however, would not dissolve readily, even at a higher dilution, and was therefore omitted. Each resuspended fraction, as well as the whole serum was sterilized by filtration through a D-8 Hormann pad in a Seitz filter.

The results (table 2) show that neither of the globulin fractions had any growth stimulatory properties, whereas the enhancement effected by albumin was as marked as that of whole serum (plate 2). Also, the two control plates strikingly contrast one another, and re-emphasize the need of an added agent in tryptose agar to permit growth of this brucella strain. Further proof of the distinctive, fastidious character of this strain was obtained in a parallel experiment, when the size attained by by the colonies of the typical, CO_2-dependent brucella strain 3038 showed no evidence of any growth-promoting effect by crystalline bovine plasma albumin, or by whole bovine serum. Furthermore, no zone of enhanced growth resulted. The similar ineffectiveness of added, killed brucella cells on the colonial development of typical strains has already been reported by Huddleson (2).

Other data not included in table 2, wherein the listed blood fractions were recombined in pairs and added to agar by paper disc, caused no enhancement, but demonstrated that a decrease in the concentration of albumin, reduced both the size of the colony and the zone of enhancement (plate 3). Because of this fact, the minimum concentration of albumin that would just support enhanced colonial development was then determined. To do this, the basic tryptose medium was prepared as usual, autoclaved, and the proper dilution of a sterile albumin suspension added to the melted, sterile agar just before pouring plates. The data obtained appear in table 3. Unfortunately, at this time the culture dissociated to a

TABLE 2

EFFECT OF BOVINE BLOOD PLASMA FRACTIONS ON COLONIAL GROWTH
(Paper disc method)

Agent Used	Concentration of Protein (percent)	Radius of Growth from Disc[a]	Colony Diameter[a]	Colony Count
Crystallized bovine plasma albumin[b]	4.8	16	1.4 - 0.1	200
Gamma globulin[c]	1.6			0
Beta globulin[d]	1.6			0
Bovine serum[b]	8.0	15	1.3 - 0.1	178
Control[e]				0
Control[e,f]			1.1 - 0.5 Av.=0.95	1200

[a] In mm.
[b] Armour and Company
[c] Fraction II, Armour and Company
[d] Fraction III-1, Armour and Company
[e] No agent added
[f] Killed Br. abortus cells (2×10^9/ml.) in agar

type which on tryptose agar of pH 7.5 displayed a low incidence of micro-colony development. Efforts to regain the previous phase type were of no avail. This, henceforth, necessitated use of a control plate of tryptose agar, pH 7.5, with each experiment in order to determine the significant minimum colony diameter on the test plates. At no time did the maximum diameter of the dissociated type colony exceed 0.6 mm. on a control plate. Unchanged, still, was the fact that no colonies developed on agar of pH 7.1 - 7.2. Colonies of the typical, CO_2-dependent 3038 strain show no such response. Reducing the pH from 7.5 to 7.1 decreases colony size no more than 0.4 mm. and has no effect on plate colony count.

Table 3 verifies the observation that colonial size decreased as albumin concentration was reduced. Even so, the lowest concentration tried, 0.0001 percent, enhanced the size of the colonies over those of the control. Since the colonial diameter of a strain of typical brucella develops in 72 hours to 1.2 - 1.3 mm., then from table 3, the 0.01 to 0.02 percent concentration is shown to be the lowest concentration of albumin that will produce normal size colonies. As the albumin concentration exceeds the optimum 0.01 to 0.02 percent range, there is no corresponding increase in colony size, as one might expect.

A further observation gained from table 3 concerns pH. As intimated in a previous paragraph, increasing the acidity of non-fortified tryptose agar diminishes the colonial diameter of the strain 119 Br. abortus until no colonies arise at all on media of pH 7.1 - 7.2; but addition of albumin to agar changes this picture. If an excess of albumin is present, little or no variation in colony size results, even though the pH be reduced from 7.5 to 7.1. A small decrease in colony size does occur, however, if the albumin concentration is less than the optimum 0.01 to

TABLE 3

EFFECT OF CONCENTRATION OF CRYSTALLIZED BOVINE PLASMA ALBUMIN IN AGAR ON COLONIAL GROWTH

Concentration (percent)	pH	Colony Diameter (in mm.)	Colony Count
0.0001	7.5	0.7	moderate[a]
0.0005	"	0.8	"
0.001	"	1.0	"
0.005	"	1.1	"
"	7.2	0.8	"
0.01	7.5	1.2	high[b]
0.02	"	1.3	"
0.05	"	1.1	moderate
"	7.1	1.1	high
0.1	7.5	1.1	low[c]
"	7.1	1.0	"
0.24	7.5	1.3	"
"	7.1	1.2	"
control[d]	7.5	0.5	"
control[d,e]	"	1.0	high

[a]700 to 1200 colonies per plate
[b]1200 to 2000 " " "
[c]300 to 700 " " "
[d]No added albumin
[e]Killed Br. abortus cells (2×10^9/ml.) in agar

0.02 percent. The weaker the concentration of albumin, the greater the decrease in colonial diameter when the medium pH is 7.1.

Similarly, though not shown in the table, albumin concentration is again a factor when comparing the procedure of adding albumin to the agar medium before autoclaving, with that of adding after autoclaving. When the albumin concentration is in excess of the optimum, the order of procedure causes no resultant variation in colony size, but when the albumin concentration is below the optimum, size variation occurs. For example, a colonial diameter of 1.0 mm. upon 0.001 percent albumin agar, diminishes to 0.6 mm. when this same amount of albumin is present during autoclaving.

Also of interest in table 3 is the unmistakable trend in colony count. This likewise bears a relationship to the concentration of albumin in the medium. Due to the inoculation procedure employed, the counts are not presented as absolute values, but because of the surprising degree of reproducibility that was apparent when the results were reworked because of the dissociation of the culture already mentioned, it was felt the singular trend deserved mention.

To determine, if possible, whether the enhancement activity of whole bovine serum could be entirely accounted for in the albumin fraction, whole bovine serum was added to agar medium in the same manner as that described for albumin. The results recorded in table 4 compare favorably with those of table 3. The lowest concentration of bovine serum required for normal colonial development of 1.2 mm. lies within the broad 0.1 to 1.0 percent range. More precisely, 0.5 percent whole serum would be the median amount of choice. This is equivalent to 0.025 percent concentration of albumin. Essentially, then, this reaffirms the optimum range set forth in table 3 and thereby strengthens the likelihood that the growth

TABLE 4

THE EFFECT OF CONCENTRATION OF WHOLE BOVINE SERUM IN AGAR ON COLONIAL GROWTH

Serum Added		pH	Colony Diameter (mm.)	Colony Count
Concentration (percent)	Albumin Concentration (percent)			
0.001	0.00005	7.5	0.3	low[a]
0.01	0.0005	"	0.4	"
0.1	0.005	"	1.0	"
0.5	0.025	"	1.2	high[b]
1.0	0.05	"	1.2	moderate[c]
2.0	0.1	"	1.3	low
5.0	0.25	"	1.3	"
Control[d]		"	0.5	"
Control[d,e]		"	1.0	high

a = 300 to 700 colonies per plate
b = 1200 to 2000 colonies per plate
c = 700 to 1200 " " "
d = no added bovine serum
e = killed Br. abortus cells (2 x 10^9/ml) in agar

promoting factor in serum is albumin. Data analogous to that in table 3, is the effect of concentration upon colonial development, and also the trend in plate colony count. Though the effect of a more acid pH was not determined, a trend like that indicated in table 3 is predicted here. One can note that the two lowest serum concentrations used have no enhancement effect.

3. The effect of egg albumin on colonial growth. Although it is well known that egg albumin is a protein distinct from serum albumin, its trial, in the substance of fresh egg white, was included as a matter of interest. Accordingly, the white from a fresh egg was added either from saline suspension, or directly to the basic ingredients of the agar medium before heating to dissolve. Egg albumin also, depending upon concentration was found to promote enhanced colonial development, as table 5 illustrates.

4. The effect of denaturation and digestion of albumin on colonial growth.

Subsequent to the conclusion that the growth-promoting action of whole bovine serum lay in the albumin fraction, attempts were made to improve the stimulatory capacity of this fraction. Since the characteristics of a protein are determined by the physical configuration or arrangement of polypeptide chains within the molecule, it seemed probable that steric rearrangement, likened to "unwinding," or disruption of these chains, would alter the growth-promoting action of albumin -- possibly intensify it. Such molecular modification, or denaturation, is not typified by an "all or none" behavior (5). That is, degrees of structural change evolve depending upon the nature of the protein and the denaturing agent used. Two methods of denaturation (physical, and chemical) and three of digestion (biological) were studied.[1]

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[1] The term, denaturation, has fallen into rather loose usage. Generally speaking the term denotes ill-defined changes in protein properties. Therefore, Neurath, et al. (5) have defined denaturation as "any non-proteolytic modification of the unique structure of native proteins, giv-

TABLE 5

EFFECT OF CONCENTRATION OF EGG WHITE IN AGAR ON COLONIAL GROWTH

Concentration (percent)	pH	Colony Diameter (in mm.)	Colony Count
0.001	7.5	0.1	<low
0.01	"	0.4	low[a]
0.1	"	0.8	"
1.0	"	1.2	high[b]
5.0	"	1.2	"
20.0	8.2	1.3	"
control[c]	7.5	0.5	low
control[c,d]	"	1.0	high

[a] 300 to 700 colonies per plate
[b] 1200 to 2000 " " "
[c] No added egg white
[d] Killed Br. abortus cells (2×10^9/ml.) in agar

The initial attempt consisted of heating sterile, saline solutions of albumin (4.8 percent) at 60°C. and at 80°C. for 30 minutes in a water bath. A colloidal, "cloud-point" suspension developed at 60°C., but due to extending the degree of denaturation, the higher temperature caused the second solution to coagulate. Though centrifugation separated supernatant from precipitate, only soluble material lends itself to assay by the filter paper disc method, so that it was impossible to learn, on a comparable basis, whether activity, if any, may have been retained, or, possibly concentrated, in the precipitate portion of the coagulated protein. Be that as it may, assay of the solution heated at 60°C. showed activity reduced by approximately one-third (plate 4), while none remained in the supernatant liquid from the 80°C.-treated specimen.

The second means of denaturing (chemical) utilized the action of hydrogen and hydroxyl ions outside the stable range of pH. This hydrolytic procedure seemed worthy of trial primarily because the solubility of bovine albumin could be maintained even though heating to a temperature higher than 80°C. Such is possible when performed in the presence of sufficient acid or alkali (pH 2 or pH 11). Although solubilizing is apparently the main effect occurring at pH 2, the soluble "sodium albuminate", after extended heating, is characterized by visible hydrolytic changes, such as an increase in viscosity and an amber coloration (6). For this reason, only a trace of alkali (pH 8) was employed. Thus, two

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ing rise to definite changes in chemical, physical or biological properties." (Omission of any reaction whereby protons are removed from or added to the native protein, is understood.) In accord with this definition, enzymic digestion is set apart from denaturation.

aqueous solutions of bovine plasma albumin (2 percent), one adjusted to pH 8, the other to pH 2, were autoclaved 30 minutes at 121°C. The pH of the alkaline solution remained constant during autoclaving and was not lowered before testing. The solution of acid pH was tested unadjusted, as well as after adjusting pH to neutrality. The results obtained from testing the three solutions showed activity reduced by nearly one-third. For example, the albumin sample autoclaved at pH 2 and tested without pH adjustment showed maximum colony size of 1.2 mm., and a radius of enhancement zone equal to 10 mm., compared with the respective values for untreated albumin (2 percent) of a 1.5 mm. colonial size, and a 15 mm. radius.

Such reduced activity may be caused by one or several changes characteristically wrought by denaturing agents. One change of a physical nature that is likely to have occurred, is that of a lowered diffusibility. When heat is involved as a denaturation agent, a decrease in diffusibility is explained by an increase in molecular size, due to a polymerization that follows intramolecular rearrangement. Polymerization that has gone to completion is visible in the form of coagulum. Yet, whereas a coagulated protein is always a denatured protein, it of course does not follow that one remaining soluble has not undergone denaturation. Cooper and Neurath (7), by diffusion, viscosity and electrophoretic measurements, showed that polymerization occurred in crystalline horse serum albumin which they had heated at the pH that maintained solubility.

There are also changes of a chemical nature within the molecule that should be considered as causes of reduced activity. In the many recent studies of Klotz and his associates concerning the chemical binding power of proteins, and especially of serum albumin, they postulate the chemical change that occurs within the albumin molecule when heat denatured,

and which is manifested by a diminished binding power, to be due to a decrease in net number of free, cationic residue groups, particularly ≡NH+ residues, remaining on the albumin molecule (8). They believe these cations responsible for the strong binding of anions to albumin. If such binding action by albumin favors growth enhancement of Br. abortus 119, then, per se, any process that reduces the available cations on the albumin molecule would also reduce the growth-promoting ability of albumin.

The third method of treating albumin, digestion by biological agents, involved the use of three proteolytic enzymes: papain, trypsin, and pepsin. Examination of the albumin fraction by digestive means was based on the principle that enzyme action -- fragmentation -- might release the physiologically active segment or segments responsible for the growth-enhancing activity of the intact albumin. Procedural modifications that will not be elaborated upon were tried before concluding the appraisal of this treatment.

The initial examination of both trypsin and papain, using a saline suspension of the crystalline bovine plasma albumin (4.8 percent), followed the procedures recommended by the manufacturer. The initial digestion procedure using pepsin, was that adopted by Huddleson and Pennell (9) to purify Brucella antiserum. Assurance that digestion had proceeded to completion was signified by failure of the albumin-enzyme mixture to coagulate when heated at isoelectric conditions for 30 minutes at 80°C. Yet, no matter how the papaic digestion procedure was modified, at no time did the reaction go to completion as determined by this criterion if begun with native protein.

It was found that the effect of the resultant enzyme-albumin digests on the growth of the fastidious Br. abortus also was a measure of the

completeness of digestion. That is, when the 30-minute heating at 80°C. caused no protein to coagulate, neither was the digest growth stimulatory, either on solid or in liquid media, as the data in table 6 and 7 indicate. On the other hand, if any coagulation occurred before termination of the heating (incomplete digestion), then the extent of digestion was reflected in the extent of the growth stimulated by the agent. Though progressive loss of growth activity as digestion proceeded to completion should have been ample evidence that this method of treating albumin was ineffectual, yet known variability in end-products obtained by use of different enzymes provoked trial of some modified procedures, especially some involving the use of papain, as already implied.

The results as set forth in table 6 are representative of data from these various modified procedures. Because papain repeatedly failed to elicit complete digestion, this enzyme received closest inspection. Beside trials with non-activated papain, both thioglycollic acid-activated and KCN-activated papain preparations were tested. The report by Rice et al. (10) that heat denaturation of protein increased the rate of papaic digestion of human serum albumin, suggested trial of a similar procedure using bovine plasma albumin. Accordingly, digestion was initiated on a soluble albumin sample (2 percent), treated previously by autoclaving at pH 8. This routine actualized complete digestion as indicated by lack of coagulum at 80°C. (table 6, agent 5).

To establish that the enzymes, alone, had no inhibitory effects on the growth of the Br. abortus strain, each one was tested by the paper disc method on agar fortified with killed brucella cells, in the concentration used for digestion. Finally, to avoid any inhibitory effects from reagents other than enzymes, these, too, were tested by placing on

TABLE 6

EFFECT OF ENZYME DIGESTED ALBUMIN ON COLONIAL GROWTH
(Paper disc method)

Agent	Conc. (percent)	Radius of Growth from Disc[a]	Colony Diameter[a]
1. Albumin, whole	4.8	16	1.4 - 0.1
2. Albumin, after 8 hr. trypsin treatment (80°C. heat-coagulated)	4.8	13	1.2 - 0.1
3. Albumin, after 20 hr. trypsin treatment (not heat-coagulated)	4.8	0[b]	
4. Albumin, after 20 hr. papain treatment (80°C. heat-coagulated)	4.8	16	1.3 - 0.1
5. Albumin, autoclaved at pH 8, followed by 11 hr. papain treatment (not heat-coagulated)	2.0	—[c]	0.6 - 0.1
6. Albumin, after 1 1/2 hr. pepsin treatment (slight coagulation at 80°C.)	5.0	10	1.1 - 0.1
7. Albumin, after 2 hr. pepsin treatment (not heat coagulated)	5.0	—[c]	0.5 - 0.1
8. Trypsin - saline solution[d]	0.1	—[c]	1.2 - 0.8
9. Pepsin - saline solution[d]	0.1	—[c]	1.2 - 0.8
10. Control[e]		—[c]	0.6 - 0.1

[a] In mm.
[b] No detectable colonies anywhere on agar surface
[c] Colonies distributed evenly over entire agar surface
No zone of inhibition or enhancement
[d] On agar fortified with killed cells
[e] No agent added

TABLE 7

THE EFFECT OF ENZYME DIGESTED ALBUMIN ON GROWTH IN BROTH

Agent Added to Broth	Conc. (percent)	Turbidity[a] 48 hrs.	72 hrs.
Albumin, whole	0.2$_4$	2+	5+
Albumin, after 20 hr. trypsin treatment (not heat-coagulated)	0.1	-	-
Albumin, after 20 hr. papain treatment (80°C. heat-coagulated)	0.1	1+	5+
Control[b]		-	-
Control[c]		2+	5+

a = degree of growth by turbidity
b = tryptose
c = trypticase soy
- = no turbidity

paper discs.

In view of the frequency with which the 4.8 percent solution of native crystalline bovine albumin was used as a control when testing samples by the filter paper disc method, four observations deserve mention here. An albumin solution (4.8 percent) retains its growth-enhancing potency after storage for as long as six months at 4°C. And activity is not influenced by a minor change in pH (that is, from the usual norm of 5.7 - 6.0 to a neutral 7.0 - 7.3), as ascertained by the disc method. Yet, increasing atmospheric CO_2 content to 10 percent reduces colonial diameters slightly, but has little or no effect on the spread of enhancement from the disc; while repassage through a Hormann D8 filtration pad understandably decreases growth-stimulatory activity because the adsorbing action of the filter pad reduces albumin concentration.

It seemed possible that by enzyme fragmentation of the albumin molecule, some nutritionally stimulating factor might be released that could later be isolated and identified. On the other hand, if potency of albumin were due to some other physiological influence, digestion probably would not be worthy of trial. However, there have been a few important instances where loss in biological activity of proteins has not followed digestion. An exemplary case of this has been the preparation of antitoxins for therapeutic use -- especially diphtheria antitoxin. Such a possibility further encouraged trial of digestion.

Choice of enzymes was based both on the fact that trypsin, papain, and pepsin are noted for their proteolytic activity, and on the fact that these three enzymes have been used successfully in the above-mentioned preparation of diphtheria antitoxin. Also adopted from this latter digestion procedure, in which non-antitoxic protein is digested away from active

antitoxin in serum globulin from the immunized horse, was the criterion of complete digestion, i.e., failure of protein to heat coagulate (11).

Measuring the extent of digestion by this arbitrary standard by no means discloses what split products have resulted from enzyme action. Because the present concept of the serum albumin molecule, either human or bovine, is that of a single polypeptide chain containing one readily available sulfhydryl group per mole (12), and one N-terminal alpha-amino group (always an aspartic acid residue) (13), a mixture of split products is reasonably expected. (On the other hand, several -SH groups per mole of egg albumin are indicated, but because these are not near the surface of the molecule they are not readily available.) Furthermore, the mixture proportions and extent of fragmentation are variable. Both the enzyme used and experimental conditions, even though only slightly modified, are known to affect final outcome. For this reason, it would seem that if any products of digestion were to have displayed growth stimulation, then the three enzymes used, and the several procedural methods of digestion tried should have provided adequate opportunity to show this, unless the final concentration of the fragmented product was too dilute.

Though interest in what end-products might be present was of secondary moment, conjecture arose as to their possible identity when indication of growth inactivity became more certain. Actually the amino acid composition of bovine plasma albumin has been well characterized from numerous studies. For instance, the four most abundant amino acids present, in decreasing order by weight, are: glutamic acid, leucine, lysine, and aspartic acid (14). Search of the literature revealed a rather surprising report by Riesen and Elvehjem (6). They report that there was only a negligible release of free amino acids from native, or heated, soluble bovine

plasma albumin when digested either by pepsin, trypsin, trypsin-pepsin, or pepsin-trypsin. Instead of amino acids, enzymatic hydrolysis resulted mostly in release of peptides, the average size of which depended upon the enzyme used. For example, pepsin-freed peptides had fewer amino acid residues than those released by trypsin. Likewise, the enzyme determined the principal amino acids contained in these respective peptides. Further proof of the individuality of enzymatic action for a given substrate was the mention that pepsin was twenty times as active as trypsin (on a weight basis) in effecting digestion of native albumin. Analogous to this finding is the notation in table 6 of the time required for digestion by pepsin compared to that needed by trypsin.

Incidentally, the improved ease with which papain attacked the soluble, heat-denatured albumin is the result of better accessibility to peptide bonds, the characteristic structural point of attack of proteases. Such predenaturation of protein, that is, intramolecular 'unwinding', whether by heat or other enzymes, improves the rate and ease of attack of most proteases, except pepsin (6) (10) (15).

5. <u>The effect on colonial development of albumin heated in the presence of sugars.</u> Since, from the above results, it is clearly apparent that degradation of the albumin molecule, either by denaturation or enzymatic digestion, does not secure the improvement sought in the growth-stimulatory capacity, there remained one alternative approach to the problem, that of heating albumin while, at the same time, by means of saturated sugar solutions, protecting against denaturation. The promise held forth by this method lay in the fact that, whereas degradation was provoked by the previous procedures, now the albumin molecule would remain intact, or nearly so, as evidenced by electrophoretic patterns made before and after

heat treatment (16). Besides this, possible formation of the colloidal 'C' component, characteristically noted in electrophoretic analyses of heated serums and plasmas, would be averted.

The investigation was confined to trial of three sugars: glucose, lactose, and sucrose. The procedure followed was patterned after that used by Hardt et al. (16, 17).

Three saline solutions of albumin (4.8 percent) were each saturated at room temperature with a sugar. Aliquots from these stock sugar-albumin solutions were heated for one hour, at 65°C. and at 80°C., in a constant temperature water bath. The appearance of each solution was noted after heating. One sample from each stock sugar-albumin solution was not heated. These served as controls. A fourth control was an albumin solution (4.8 percent), containing no sugar, heated at 65°C. for one hour. In this latter control a very fine, nearly colloidal coagulum formed which was later removed by centrifugation. All heated samples and all controls were dialyzed 72 hours against physiological saline. The final volume and pH of each solution was recorded after dialysis. In the case of glucose, assurance of complete sugar removal was confirmed by Benedict's test for reducing sugar. Finally, each solution was sterilized by filtration through a Hormann D8 pad in a Seitz filter, and tested for growth-stimulatory activity by the paper disc method.

The characteristic appearance of each sample after heating apparently depended on the saturating sugar. All non-heated samples were water-clear, and there was no change from this initial appearance for either glucose or sucrose samples heated at 65°C. But at 80°C., both samples slowly developed an opalescence and a slight viscosity and gel. However, the lactose saturated sample heated at 65°C., developed an opalescence almost

at once, while the 80°C. temperature further proved lactose a poor heat-protecting sugar. That is, a dense, white coagulum precipitated immediately and rendered further procedures on this sample useless.

The pH of the samples varied from 5.74 for the albumin control heated at 65°C. in the absence of sugar, to 7.0 for the lactose-albumin sample heated at 65°C. No adjustment of pH was made.

No adjustment of protein concentration was made following dialysis. The weakest albumin concentrations occurred in the samples previously saturated with sucrose. These concentration values ranged from 0.72 percent to 0.78 percent. In the specimens from which glucose and lactose had been dialyzed, albumin concentrations varied from 1.78 percent to 1.92 percent. Yet when testing these various samples, it was necessary to consider only the greater dilution of albumin in the sucrose-treated samples, since it was known from previous trial by the paper disc method, that a content reduction from 4.8 percent to 2.0 percent albumin elicited no modification in total growth enhancement.

The inclusive results obtained by this heating procedure showed that the process neither augmented nor diminished the growth-enhancement action of albumin on Br. abortus 119. (table 8).

Failure of albumin to show an altered capacity in its growth stimulation of the fastidious brucella strain subsequent to heat treatment in the presence of saturated sugars, may be accounted for by any one, or, combination, of the following three suppositions: 1) heat exposure evoked no appreciable intramolecular change in the albumin molecule, which is to say, that protection by sugars against denaturation was sufficiently complete to avert any responsive structural change that in turn would reflect altered growth promotion of Br. abortus 119; 2) the extent of regeneration

TABLE 8

THE EFFECT ON COLONIAL GROWTH OF ALBUMIN HEATED IN SOLUTIONS SATURATED WITH SUGAR
(Paper disc method)

Albumin Plus Sugar Used	Treatment Temperature (°C.)	Colonial Growth[a]
No sugar	65°	slight decrease in colony size and enhancement area
Glucose	rm. temp.	a like response
"	65°	" " "
"	80°	colony size same, enhancement area smaller
Sucrose	rm. temp.	decrease in colony size and enhancement area[b]
"	65°	slight decrease in colony size and enhancement area[b]
"	80°	colony size same, enhancement area smaller[b]
Lactose	65°	a like response
"	80°	not tested

a = results expressed on comparative basis, i.e., with the colonial growth obtained using 4.8% unheated albumin
b = lowered albumin concentration (percent)

of albumin from the denatured state was adequate to show an almost unchanged growth response; 3) by the suppression of 'C' component formation, there was no concomitant reduction in the amount of free albumin.

As mentioned before, denaturation consists of two consecutive reactions: structural unfolding within the molecule, followed by aggregation or polymerization. Of these, unfolding, by far the more important action, is the essence of denaturation. But the term, thermal stability, here pertains only to the visible manifestation of denaturation, and especially to the initial stages noted as opalescence or cloud formation -- Tyndall effects. The degree to which sugars raise the thermal stability of proteins depends upon both the concentration of sugar and concentration of protein. A sugar at half-saturation permits more extensive denaturation (16). Likewise, an increase in protein concentration lessens the efficiency of the sugar action. Other factors, too, affect the protective action of sugars: length of heating, temperature, pH, sugar utilized, and the protein in question (16, 18).

Sugars are not equally efficient in their protection against denaturation. In the results, this was visually noted. Hardt et al. (16), proved such a difference electrophoretically, while Ball et al. (19), demonstrated differences in sugar efficiency by measuring the content of sulfhydryl groups in egg albumin heated in the presence of sugars. Apparently glucose, fructose, and sucrose are the most effectual. The reason for this seems related to the solubility and osmotic property of the added sugar. For instance, using equal volumes of a single concentration of albumin, saturation with lactose required 3 gms., as compared with the need for 19 gms. and 21 gms. of glucose, and sucrose, respectively. Similarly, comparison of saturation concentrations of two sugars and a sugar alcohol

used to protect egg albumin from heat denaturation (19), indicate a high degree of protection from glucose (4.6 M at saturation) and fructose (5.0 M at saturation), but poor protection from mannitol (0.8 M at saturation).

Supposition #2 presupposes that denaturation has occurred to some extent. Denatured protein that regains partial or complete solubility in water or saline, is termed regenerated protein. This process of reversion, or restoration, proceeds more readily during cooling or dialysis, and the extent of recovery of many of the properties characterizing the native protein, particularly solubility, depends partly on the extent of denaturation. In general, the milder the denaturation conditions, the more likely will reversion occur (20). The treatment adopted here, of heating while protecting against denaturation, would be classed as the mildest of denaturation conditions, so that regeneration is not unlikely, both at 65°C., and also at 80°C. where opalescence was noticeable in both sucrose and glucose saturated samples. Furthermore, the study of reversibility by electrophoresis has in many instances disclosed that regenerated protein may exhibit a mobility identical with that of the native protein (21). Such was found true for bovine serum albumin denatured with 8 M guanidine hydrochloride, a treatment more drastic than the heat denaturation used in this investigation.

The 'C' component mentioned in supposition #3 was discovered by electrophoretic study of heat denatured protein (22). Instead of a pattern that revealed greater heterogeneity than did native protein, as is the usual result of denaturation, the formation of 'C' component is an example of increased electrophoretic homogeneity accompanying heat denaturation. That is, component proteins within a heated protein mixture, may, at their own expense, form into a colloidal aggregation-complex. This is the 'C'

component (22) of the electrophoretic pattern. The mixed proteins taking part in this aggregate formation may be unrelated in nature, such as the alpha casein and gamma globulin that Kreeji (23) heated together, or they may be the component fractions of a single serum (22), or plasma (16).

Actual confirmation of 'C' component development in a heated, pure protein, such as the bovine albumin fraction used in this study, has not been established. The purest fraction of bovine plasma that Hardt et al. (16) studied consisted of albumin and alpha globulin, and exhibited 'C' component formation when heated one hour at 65°C. When Van Der Scheer and associates (22) heated a single protein, the albumin fraction from horse serum, no such complex was elicited. But it should also be pointed out that two forces in the solutions, pH and ionic strength, control the nature and magnitude of change in the electrophoretic picture of the denatured protein molecule, with the result that both the proportion of components formed, and their mobilities may be varied (7). Nevertheless, whether or not 'C' component specifically forms, suppression of heat denaturation by sugar serves to minimize formation of heat-denatured products. As a result, the concentration of the intact albumin molecule remains unchanged.

The fundamental chemistry of the protective action by which sugars, neutral solutes, suppress heat denaturation of albumin is still not satisfactorily explained (24). Ball et al. (19), discounted formation of a sugar albumin complex. But recently a report by Mohammad et al. (25) suggested that glucose adds reversibly to the amino groups of bovine serum albumin, since, if the protein is first acetylated so as to mask the amino groups, and then treated with glucose, no 'browning' reaction occurs. Ordinarily the 'browning' that appears in a heated glucose-protein solution

is apparently dependent on free $-NH_3$ groups.

Similarly, in his extensive studies of albumin, Klotz (26) revealed that fatty acid anions add to albumin at the $\equiv NH^+$ sites. But, if first denatured, albumin does not bind fatty acids. Also, like sugars, when fatty acids are bound to albumin they exert a protective action against denaturation, in fact are more efficiently protective than sugars. For instance, sodium caprylate (0.3 M) prevented coagulation of albumin at 100°C., whereas glucose (0.3 M) could not raise the thermal stability of albumin higher than 65.1°C. (27).

The binding of fatty acids by albumin has been recognized, too, from a biological standpoint by Davis and Dubos (28). They asserted that in the liquid media developed for growth of tubercle bacilli, the function of albumin is protective rather than nutritive. That is, because oleic acid contaminates the synthetic, commercially impure Tween 80 utilized in the media, a bacteriostatic effect on the growth of small inocula results unless albumin is included in the medium. Similarly, they too, found that the protection afforded a small inoculum by the presence of albumin was lost if the albumin was first denatured.

Cohn (29) reported that one of the several functions of albumin in blood is its interaction with a variety of smaller molecules such as fatty acid anions. For these it acts as transporting agent, and also serves to reduce any hemolytic action toward erythrocytes (30).

If then, this binding of fatty acid is one of the natural functions fulfilled by albumin, and relevant to the explanation of stimulated bacterial growth in one report already mentioned, why could not albumin be functioning similarly in each instance in this study in which growth-enhancement occurred in the presence of albumin?

6. The effect of two wetting agents on colonial growth. Before directly seeking the answer to this question, interest was aroused over the possibility of whether the above-mentioned Tween 80, utilized by Dubos in the liquid media supporting growth of tubercle bacilli, would similarly display a growth-promoting effect on the fastidious Br. abortus 119. This possibility came to mind since, if the added albumin was functioning protectively rather than nutritively in Dubos' medium, Tween 80 might either be acting in a manner similar to albumin (i.e., protectively), or else replacing some natural growth factor in the oft-used egg-base media in which egg albumin is a major component.

Accordingly, Tween 80 (the polyoxyethylene derivative of sorbitan monooleate), with a viscosity and color similar to that of honey, was diluted in distilled water, with which it is readily miscible, and calculated quantities added to tryptose agar before autoclaving, as indicated in table 9. From this table it may be seen that colonial development improved favorably in a manner analogous to that resulting from addition to agar of bovine albumin (table 3), or egg albumin (table 5), and, likewise, depending on agent concentration.

Also available for an analogous trial was the wetting agent, Span 80 (sorbitol esterified with oleic acid), and structurally different from Tween 80. The Tween products are ester-ethers, and derived from the Span agents by the addition of polyoxyethylene chains at the sites of non-esterified hydroxyl groups. Substitution at these -OH sites yield water-soluble products, whereas the Span-type materials tend to be water insoluble. Due to this characteristic water insolubility, those dilutions added to tryptose media before autoclaving were prepared from a warmed emulsion of water-Span 80. Tiny globules of Span appeared on the hardened surfaces

TABLE 9

THE EFFECT OF CONCENTRATION OF TWEEN 80[a] IN AGAR ON COLONIAL GROWTH

Concentration (percent)	pH	Colony Diameter (in mm.)	Colony Count
0.001	7.4	0.5	moderate[b]
0.005	7.35	0.7	high[c]
0.05	7.4	1.0	"
0.1	7.4	1.2	moderate
control[e]	7.5	0.5	low[d]
control[e,f]	7.5	1.0	high

a = polyoxyethylene derivative of sorbitan monooleate, Atlas Powder Co.
b = 700 to 1200 colonies per plate
c = 1200 to 2000 " " "
d = 300 to 700 " " "
e = no added Tween 80
f = killed Br. abortus cells (2×10^9/ml.) in agar

of those plates containing the higher concentrations.

As may be seen in table 10, colonies of the fastidious brucella strain were inhibited by all concentrations of Span except the lowest, which permitted colonial development to be no greater than 0.2 mm. in diameter, a size even smaller than those colonies arising on the control plate. In sharp contrast, Span had no inhibitory effect on either number or size of the aerobic Br. abortus 2308 colonies.

The inhibitory effect of Span just noted, recurs in the data recorded in table 11, in which the effect of the three agents, albumin, Tween 80, and Span 80, on the colonial growth of both the aerobic and the CO_2-dependent brucella strains was comparatively evaluated by the paper disc method. By means of colonial development, the results point out the contrast in the wetting agents, Tween 80 and Span 80, as well as in the two Br. abortus strains. Development of the aerobic strain 2308 responded similarly to all three agents. In each case colonies were evenly distributed over the surface of the agar, and were quite constant in size, whether adjacent to the disc pad or at the edge of the plate. On the other hand, strain 119 was again inhibited by Span 80, but the growth enhancement action of both albumin and Tween 80 was reaffirmed; the action of albumin was apparently more effective, but only by a margin that might readily yield to use of an increased concentration of Tween 80.

These three agents were next placed on agar fortified with killed brucella cells, and known to support normal colonial development of the fastidious 119 strain (table 12). By this means it was thought possible to demonstrate that the fastidious strain would develop colonies in the presence of these agents just as had the aerobic strain on the non-fortified agar. The outcome was that colonies were distributed evenly over the plate,

TABLE 10

THE EFFECT OF CONCENTRATION OF SPAN 80 IN AGAR ON COLONIAL GROWTH OF CO_2-DEPENDENT AND AEROBIC BR. ABORTUS

Concentration[a] (percent)	pH	Colony Count	
		Strain 119	Strain 2308
0.0001	7.20	very low[b]	high[c]
0.001	7.23	0	"
0.01	7.3	0	"
0.1	7.24	0	moderate[d]
Control	7.5	very low[b]	high[c]

a = expressed on a liquid basis
b = 70 to 100 colonies per plate
c = 1400 to 2000 colonies per plate
d = 1000 colonies per plate.

TABLE 11

THE EFFECT OF THREE AGENTS ON THE COLONIAL GROWTH OF A CO_2-DEPENDENT AND AN AEROBIC *BR. ABORTUS*
(Paper disc method)

Colonial Strain	Agent on Paper Disc	Zone Type	Zonal Radius[a] from Disc	Colony Diameter[a] Next to Disc	Colony Diameter[a] At Plate's Rim
119	Albumin (4.8%)	Enhancement	18	1.4 - 0.1	0[b]
	Tween 80 (10%)	Enhancement	14	1.2 - 0.1	0
	Span 80 (10%)	Inhibition (complete)	35	0	0
	Control			0.6 - 0.1	
2308 (aerobic)	Albumin (4.8%)	*		1.1 - 1.0	1.1 - 0.9
	Tween 80 (10%)	*		1.2 - 0.9	1.3 - 1.0
	Span 80 (10%)	*		1.0 - 0.8	1.2 - 1.0
	Control			1.2 - 0.9	

a = in mm.
b = no detectable colonies
* = colonies distributed evenly over agar surface

TABLE 12

THE EFFECT OF THREE AGENTS ON COLONIAL GROWTH UPON TRYPTOSE AGAR FORTIFIED WITH KILLED BRUCELLA CELLS

(Paper disc method)

Agent on Paper Disc	Zone Type	Zonal Radius[a] from Disc	Colonial Diameters	
			Next to Disc	At Plate's Rim
Albumin (4.8%)	*		1.4 - 1.0	1.2 - 0.9
Tween 80 (10%)	Sl. enhancement	5.0	1.2 - 1.0	1.0 - 0.7
Span 80 (10%)	Inhibition	5.0	0[b]	0.1 - 0.9
Control			1.1 - 0.7	

a = in mm.
b = no detectable colonies
* = colonies distributed evenly over agar surface

although those adjacent to the pad containing albumin, showed a very slight enlargement. In table 12, the notation, slight enhancement, for Tween 80, indicates that colonies tended to cluster more densely in the region of the pad. Span 80, however, still inhibited colonial development, though on this medium such action was neutralized and ultimately overcome as diffusion in agar reduced the Span concentration. Since both albumin and Tween 80 have been shown to promote colonial development, it is not surprising that on fortified medium, this effect was masked. Yet, regardless of whether the fortified agar permitted normal colonial development or not, inhibitory action of Span persisted. This result is further evidence of a fundamental difference underlying strains 119 and 2308.

7. The effect of two fatty acids on colonial growth. In the extensive studies on the synthetic Tween and Span products by Dubos and co-workers, reference is frequently made to the fact that fatty acids gradually form in these agents by hydrolysis, and as a result, manifest an inhibitory effect on small inocula of tubercle bacilli. Because of this report, and the unanswered question concerning the essence of growth-enhancement action by albumin, determination of the effect of fatty acids, alone, on the colonial growth of Br. abortus 119 and 2308 was next undertaken.

From aqueous stock solutions, the sodium salts of the organic acids chosen for test, lactic and oleic, were added to the agar medium before autoclaving. For comparative purposes, the final concentration of the two acids is presented in terms of molarity (table 13), since at room temperature one salt is a liquid, the other a solid.

Again in table 13, a comparison is made of the two brucella strains, and a colony count fluctuating in response to variation in pH is again

TABLE 13

THE EFFECT OF TWO ORGANIC ACIDS IN AGAR ON COLONIAL GROWTH OF A CO_2-DEPENDENT AND AN AEROBIC BR. ABORTUS

Agent	Concentration	pH	Colony Count	
			Strain 119	Strain 2308
Sodium lactate	1.07×10^{-1}M.	7.06	0	high[d]
	1.07×10^{-2}M.	7.45	low[b]	"
	1.07×10^{-2}M.	7.06	very low[a]	"
	1.07×10^{-3}M.	7.38	" "	"
	1.07×10^{-4}M.		" "	"
Sodium oleate	6.6×10^{-3}M.			0
	3.28×10^{-3}M.			moderate[c]
	3.28×10^{-4}M.	7.48	0	high[d]
	1.64×10^{-4}M.		0	"
	3.28×10^{-6}M. (1 micro-gm./ml)	7.06	0	"
	3.28×10^{-7}M	7.48	low[b]	"
	3.28×10^{-8}M.	7.2	very low[a]	"
Control		7.5	" "	"

a = 70 to 300 colonies per plate
b = 300 to 500 " " "
c = 725 " " "
d = 1400 to 2000 " " "

noted. The aerobic strain was not inhibited by any test concentration of lactic acid. Though the CO_2-dependent strain was finally inhibited by a 0.107 M lactic acid concentration, colonies may even have developed at this molarity had the growth-favoring pH of 7.5 also been tried, as may be concluded from the pH-variable colony count recorded for the 1.07×10^{-2} M concentration. On the contrary, oleic acid, while still very dilute, was inhibitory to both brucella strains. Still, the aerobic culture tolerated a concentration of this fatty acid exceeding by approximately 2000 times that dilution effecting inhibition of the fastidious strain. And for this acid also, a more alkaline pH favored a numerical increase in colony count of strain 119 even though oleic acid concentration simultaneously increased ten-fold, from 3.28×10^{-8} M, to 3.28×10^{-7} M.

Tables 14 and 15 report the effect on strain 119 of oleic and lactic acid, respectively, when assayed by means of the paper disc method. Dilution both by absorption on the paper disc and by diffusion into agar caused this method of assay to show toleration of a higher concentration of both these acids than was recorded for the method in which each acid was incorporated directly in the medium (table 13). The effect of pH is apparent in these two tables, also. When either fatty acid was at the threshold of the inhibitory concentration, the degree of inhibition was modified by pH, so that an alkalinity increase from pH 7.2 to pH 7.5 promoted both enlargement of colonial diameter and development of colonies where none would otherwise have appeared. Accordingly, in table 15, complete inhibition of strain 119 by undiluted lactic acid is probable had the medium also been tested at pH 7.1.

TABLE 14

THE EFFECT OF MEDIUM COMPOSITION AND OF CONCENTRATION OF SODIUM OLEATE UPON COLONIAL GROWTH

(Paper disc method)

Sodium Oleate, Concentration Placed on Disc	Agar pH	Tryptose Agar		Tryptose Agar Fortified with Killed Brucella Cells	
		Zone Type	Colonial Diameter (in mm.)	Zone Type	Colonial Diameter (in mm.)
3.28×10^{-6}M. (1[d])	7.5	*	0.1 x 0.6		
3.28×10^{-5}M. (10[d])	7.5	*	0.1 - 0.5	*	0.5 - 1.0
6.56×10^{-5}M. (20[d])	7.2	*	0.1 - 0.5	*	0.5 - 1.1
1.31×10^{-4}M. (40[d])	7.5	* and slight inhibition	0.1 - 0.3[a]	*	0.6 - 0.9
1.64×10^{-4}M. (50[d])	7.2	complete inhibition	0[b]		
1.64×10^{-4}M. (50[d])	7.5	slight inhibition[c]	0.1 - 0.4[a]		
1.97×10^{-4}M. (60[d])	7.5			* and slight inhibition	0.6 - 0.7[a]
2.30×10^{-4}M. (70[d])	7.5	moderate inhibition[c]	0.1 - 0.2[a]	* and slight inhibition	0.2 - 0.7[a]
3.28×10^{-4}M. (100[d])	7.5			* and moderate inhibition	0.2 - 0.4[a]
Control	7.5	*	0.1 - 0.6	*	0.5 - 1.2

a = in 3 mm. radial zone from disc
b = no detectable colonies
c = very few colonies beyond the 3 mm. radius from disc
d = micrograms/ml.
* = colonies distributed evenly over agar surface

TABLE 15

THE EFFECT OF pH AND CONCENTRATION OF SODIUM LACTATE ON COLONIAL GROWTH

(Paper disc method)

Sodium Lactate Concentration Placed on Disc	Agar pH	Zone Type	Zonal Radius[a] From Disc	Colonial Diameters[a] Next to Disc	Colonial Diameters[a] At Plate's Rim
1.07×10^{-3} M.	7.5	*		0.7 -<0.1	0.7 -<0.1
1.07 M.	7.5	Slight Inhibition		0.2 -<0.1	0.8 - 0.1
1.07 M.	7.23	Inhibition	4.0	0[b]	0.4 -<0.1
10.7 M (undiluted)	7.5	Inhibition	25.0	0[b]	0.8 - 0.1
Control	7.5	*		0.1 - 0.6	

a = in mm.
b = no detectable colonies
* = colonies distributed evenly over agar surface

Where a degree of inhibition is indicated in table 14, the accompanying recorded colonial diameters pertain only to colonies arising within 3 mm. of the disc. At distances beyond this, colonies increased in size to the maximum diameter recorded for the control plate.

The use of oleic acid on two agar types, tryptose and tryptose fortified with killed brucella cells is contrasted in table 14. On tryptose agar, the maximum oleic acid concentration without a growth-inhibitory effect was approximately 30 microgms./ml.. A parallel effect on tryptose agar fortified with killed brucella cells was demonstrable, even after further increasing the oleic acid concentration by 20 microgms./ml.. On the other hand, complete inhibition of colonial development on either agar type, similarly required a difference in total added oleic acid of nearly 20 microgms./ml..

Because tables 12 and 14 indicate that the sensitivity of culture 119 in the presence of Span 80 and oleic acid is modified by the use of agar fortified with killed cells, the effect of other growth-stimulatory agents upon these two growth-inhibitory agents was sought. The procedure was revised so that an inhibiting agent was incorporated in the agar and an enhancing agent was added to the paper disc. Results are recorded in tables 16 and 17 for the CO_2-dependent strain 119, and in table 18 for the aerobic culture 2308.

In the data in the first of these three tables (table 16), it is readily seen that an increasingly more dilute inhibiting agent permitted both enhancing agents to effect an increasingly wider zone of growth. In fact, enhancement was largely confined to the broadening of the zone of colonial development, since there was but slight variation in maximum colony size. Assurance that the utilized concentrations of Span 80 and

TABLE 16

THE EFFECT OF ALBUMIN AND TWEEN 80 ON COLONIAL GROWTH ON AGAR CONTAINING GROWTH-INHIBITING AGENTS (Paper disc method)

Inhibiting Agent in Agar	Enhancing Agent Added				No Enhancing Agent Added[d]
	Albumin (4.8%)		Tween 80 (10%)		
Concentration	Enhancement Zone Radius[a] From Disc	Colonial Diameters[a] in Enhancement Zone	Enhancement Zone Radius[a] From Disc	Colonial Diameters[a] in Enhancement Zone	Colonial Diameters[a]
Span 80:					
0.1 %[c]	9	1.3 - <0.1	6 (halo)	1.2 - 0.3	0[b]
0.01 %[c]	17	1.2 - 0.1	15 (halo)	1.2 - 0.1	0
Sodium Oleate:					
3.28×10^{-3} M.	2	1.0 - 0.1	2	0.6 - <0.1	0
1.64×10^{-4} M.	3	1.1 - 0.1	4	0.8 - 0.1	0
3.28×10^{-6} M.	18	1.4 - <0.1	16	1.1 - <0.1	0
3.28×10^{-7} M.	17	1.3 - 0.1	14	1.1 - 0.1	0.5 - <0.1
Control	15	1.4 - 0.7	13	1.1 - 0.7	0.7 - 0.1

a = in mm.
b = no detectable colonies
c = expressed on liquid basis
d = colonies distributed evenly over agar surface

oleic acid were truly growth inhibitory was verified by lack of colonies when no enhancing agent was added to the disc.[1]

Comparison of agents Tween 80 and albumin revealed that the latter consistently promoted growth of slightly larger colonies. This was reaffirmed in the results of table 17, but besides this there was also a dissimilarity revealed -- one identified with pH. Thus the radius of enhanced growth actuated by albumin, fluctuated sensitively with pH except when interacting with the threshold concentration of lactic acid. At this latter point, similar, wide zones of growth occurred both at pH 7.1 and pH 7.5 . On the other hand, analogous examination of Tween 80 reveals that change in neither pH nor lactic acid concentration caused significant fluctuation in zone size. In the light of these results, though both agents demonstrate ability to counteract growth-inhibitory substances, essential differences are intimated in the manner by which each agent accomplishes this antagonistic effect.

Prior to, and underlying such counter-action is the mechanism of inhibition. Whether inhibition is due to a vital effect occurring at the cell wall (such as altered permeability), whether achieved because of a chemical influence, or whether it is the result of a transformation in cellular metabolism, remains conjecture (31), since such phenomena are admittedly complex in nature. Generally, the physico-chemical theory is favored (32), as reiterated recently in a review by Nieman (33). This

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[1] During the course of work with Span and Tween, an interesting result was observed when Tween was added to Span-agar via the paper disc. After diffusion into the medium, a cloudy-white, halo-like ring developed in the agar in that area surrounding the disc pad where the proper ratio of Tween:Span occurred. Diminutive 'halo' formations also surrounded the larger Span globules when located sufficiently near the disc pad. Probably colloidal in nature, this was perhaps a manifestation of reversal-of-emulsion-type.

TABLE 17

THE EFFECT OF pH AND OF ALBUMIN AND TWEEN 80
ON COLONIAL GROWTH ON AGAR WITH AND
WITHOUT SODIUM LACTATE CONTENT
(Paper disc method)

Agar Medium		Enhancing Agent Added				No Enhancing Agent Added[c]
		Albumin (4.8%)		Tween 80 (10%)		
Sodium Lactate Concentration	pH	Enhancement Zone Radius From Disc[a]	Colonial Diameters in Enhancement Zone[a]	Enhancement Zone Radius From Disc[a]	Colonial Diameters in Enhancement Zone[a]	Colonial Diameters[a]
absent	7.5	15	1.4 - 0.7	13	1.1 - 0.7	0.6 - 0.1
absent	7.1	10	1.2 - 0.1	11	1.0 - 0.7	0[b]
1.07×10^{-2} M.	7.45	20	1.4 - <0.1	14	1.2 - 0.1	0.6 - 0.1
1.07×10^{-2} M.	7.06	19	1.4 - 0.1			0.2 - 0.1
1.07×10^{-3} M.	7.38	14	1.4 - 0.1	12	1.1 - 0.1	0.3 - 0.1

a = in mm.
b = no detectable colonies
c = colonies distributed evenly over agar surface

concept envisions that agents such as fatty acids are adsorbed at the cell membrane, so that growth interference, as well as any stimulatory effect, is the result of a subsequent change in cell permeability.

The counter-action of albumin, long recognized as a protective colloid, must be to remove the offending organic acids from effective contact with the bacterial cell by adsorption -- an interaction utilizing both electrostatic and non-electrostatic forces (26, 34, 35). Regardless of the resultant protein-organic acid complex, however, an increasingly more acid environment exerts a growth-hindering effect on the fastidious strain 119, as already shown.

Tween 80, on the other hand, is not protein in nature, but rather a water miscible, non-ionic, surface-active, synthetic compound, whose actual effect on bacterial growth is based on conjecture. If, as expected, its behavior is that of an emulsifying agent, then, figuratively speaking, Tween 80 interjects itself between the organic acid and the bacterial cell so as to form a protective film, the emulsifying film. This would both shut out and remove the offending organic acid molecule to a further distance from the cell (36). Moreover, basis for such behavior has been suggested by the electrophoretic results obtained using Lactobacillus casei in the presence of a non-ionic agent (37). The mobility measurements showed a decrease and finally stabilization, thereby indicating adsorption of detergent on the surface of the cells. Such a non-ionic envelope could, in a like manner, serve to modify the growth-restraining effect of an increasingly more acid environment.

Though Span 80 is also a non-ionic, synthetic detergent and esterified with oleic acid, it is not, however, water-miscible like Tween 80, and therefore cannot act as an emulsifying agent. Because of this hydrophobic

nature, Span 80 does not function protectively toward bacterial cells, as may be construed from the intact globules noted throughout the agar medium. The observed inhibitory action is attributed to this immiscibility, along with the accompanying presence of unesterified oleic acid that has accumulated slowly in the Span compound as a result of hydrolysis (32, 38, 39). Though a similar slow, hydrolytic breakdown also occurs in Tween 80, because of its emulsifying capabilities, any inhibitory effect is delayed until the free fatty acid concentration passes into the range critical for cell reproduction. Since albumin, killed brucella cells, and Tween 80 can, depending on concentration, overcome the inhibitory action of oleic acid (tables 14 and 16), so, likewise, can these agents counteract the inhibitory action of Span (tables 12 and 16), per se a reversal of oleic acid inhibition.

In summary then, the results in tables 16 and 17 are determined by three factors: the concentration of enhancing agent, the pH of the medium (table 17, only), and the ratio of enhancing agent to inhibiting agent. The result of maintaining concentration and pH constant, demonstrates that sensitivity of the CO_2-dependent Br. abortus toward fatty acid or Span 80, when either completely inhibitory (oleic acid), or only partly so (lactic acid), can be overcome by the action of albumin or of Tween 80, and the extent of this neutralizing action is essentially a function of the ratio of lipid:agent. Furthermore, there apparently is an optimal ratio-combination that stimulates growth to an extent better even than that resulting from the enhancing agent used alone, or in combination with a greater or lesser amount of organic acid.

Unlike strain 119, the aerobic Br. abortus 2308 has indicated a growth-sensitivity neither to the agents used in table 11, nor to tryptose

agar in the absence of these agents. Yet, the data recorded in table 18 show that the colonial development of this strain can be just as responsive as the fastidious 119 strain to Tween 80 and albumin. A growth-sensitive behavior was induced by incorporating in the agar medium that quantity of sodium oleate known from table 13 to cause partial growth inhibition; that is, decrease in colony size and number, and reaffirmed by #1 in this table. Following incorporation of this concentration of sodium oleate in agar, growth enhancement was noted when either albumin or Tween 80 was added to the paper disc (#2 and #3), but for the first time, Tween 80 here outperformed albumin. Nevertheless, increasing the concentration of Span in agar beyond that previously tried (table 11), by placing Span in the basal medium as well as upon the disc pad (#6), still provoked no growth-inhibitory response from the aerobic strain. Thus, it can be deduced that though oleic acid contaminates Span 80, the accompanying concentration must be less than 0.00328 M, since this quantity induced partial growth inhibition (#1) in agar medium.

By way of contrast, a growth-inhibitory response was finally obtained for strain 2308, when sodium oleate was added to disc pad as well as to medium (#4), just as was done for Span. However, the inhibition obtained by this procedure did not far surpass that resulting from addition of the same molar concentration of lipid to disc pad only (#5), on control medium. In fact, because of the high soap concentration in these two instances (#4 and #5), the resultant inhibition may actually have stemmed from antiseptic or disinfectant action.

The following is a comparative review of the results thus far obtained for the two Br. abortus strains 119 and 2308, when each is exposed to the respective threshold inhibitory concentrations of fatty acid in agar medium:

TABLE 18

THE EFFECT OF SEVERAL AGENTS ON COLONIAL GROWTH OF AN AEROBIC BR. ABORTUS ON AGAR CONTAINING GROWTH-INHIBITORY AGENTS

(Paper disc method)

	Inhibiting Agent and Concentration in Agar	Agent Added to Paper Disc	Zone Type	Zonal Radius[a] From Disc	Colonial Diameters[a]	
					Next to Disc	At Plate's Rim
	Sodium Oleate:					
1.	3.28×10^{-3}M.	No agent	*		1.2 - 0.7[e]	
2.	"	Albumin (4.8%)	Enhancement	5.0	1.6 - 1.4	0.6 - 0.3
3.	"	Tween 80 (10%)[c]	Enhancement	10.0	1.4 - 1.1	0.6 - 0.3
4.	"	Na Oleate (3.28×10^{-2}M.)	Inhibition	6.0	0[b]	1.1 - 0.6
5.	Control[d]	Na Oleate (3.28×10^{-2}M.)	Inhibition	5.0	0[b]	1.2 - 0.8
6.	Span 80: 0.1%[c]	Span 80 (10%)[c]	*		1.4 - 0.9	1.4 - 0.9
	Control[d]	No agent	*		1.4 - 1.0[e]	

a = in mm.
b = no detectable colonies
c = expressed on liquid basis
d = no added agent in agar
e = colony count, moderate (700 - 1050)
* = colonies distributed evenly over agar surface

	Br. abortus #119	Br. abortus #2308
Sodium oleate:		
threshold inhibitory concentration in agar medium ...	3.28×10^{-7} M	3.28×10^{-3} M
Diameter of enhancement zone effected by: albumin, 4.8% ..	17 mm.	5 mm.
Tween 80, 10% .. added to disc pad on agar containing above sodium oleate concentration	14 mm.	10 mm.
Colonies beyond enhancement zone ..	absent	present
Sodium lactate:		
threshold inhibitory concentration in agar medium ...	1.07×10^{-2} M	none established

Furthermore, the data have also established that four agents -- bovine serum, albumin, Tween 80, and killed brucella cells, promote growth of both brucella strains. Inhibition of the colonial development of Br. abortus 119, on the other hand, has been accomplished by both lactic acid and oleic acid (mentioned in order of increasing toxicity). The aerobic Br. abortus 2308 is apparently less sensitive to these toxic agents since only oleic acid of higher concentration has evinced inhibitory action for this strain.

The reversibility of both lactic and oleic acid toxicity has also been recognized. Reversion has resulted from either one of two procedures: reduction of effective concentration of organic acid; or introduction of a suitable agent (detoxifier), whose role may be one of complex-formation with the fatty acid, or, figuratively speaking, competitive adsorption (between agent and fatty acid) at the cell wall (33). Moreover, toxicity reversal has been noted for fatty acids inhibitory toward other bacterial

species, among which the gram positive lactobacilli are a prime example (31). Dubos (39, 40) likewise indicated the same effect on tubercle bacilli, and whether the fatty acid action proved inhibitory or stimulatory depended upon such experimental conditions as fatty acid concentration, size of cell inoculum, and length of incubation before growth measurement.

Finally, not only has reversal of toxicity been noted, but the presence of a certain ratio of fatty acid to reversing agent is apparently growth stimulatory. For example, in table 16, the radius of zonal growth obtained for either albumin or Tween when interacting with sodium oleate of 3.28×10^{-6} M concentration is wider than that obtained for either respective control (either agent alone), or for the more dilute fatty acid concentration. Likewise, in table 17 an enlarged zonal radius occurs when the lactic acid concentration is 1.07×10^{-2} M.

Such a stimulated growth response in the presence of a particular concentration ratio of agent (especially a protein) and fatty acid, is not unique to this study alone. A similar situation was described by Oyama et al.(41) for the cultivation of the Reiter treponeme, for which the optimal growth-stimulatory response for the protein:lipid ratio depended quantitatively on the lipid concentration, a variable contingent on the fatty acid in question, and these workers have devised a graphic means of determining the optimal ratio. Growth was promoted neither by a ratio containing too little fatty acid, nor by one containing an excess. On the other hand, when the lipid concentration was maintained constant in the non-toxic range, and the concentration of protein (crystallized bovine plasma albumin) was varied over a wide gamut, the resultant quantity of growth varied but slightly.

In another instance, Tomarelli (42) reported that various fatty acids exerted diverse growth effects upon two forms, the bifidus and the rod, of the gram positive Lactobacillus bifidus. But more pertinent, the toxicity of those fatty acids found to be inhibitory, including oleic acid, could be alleviated by use of several agents, including bovine serum albumin and the surface active agents Tween 80, and Tween 60. In fact, an excess amount of oleic acid, inhibitory only for the bifid form, heightened growth-stimulation of both strains if utilized in a certain ratio with Tween 80. This growth response surpassed that evoked by either Tween, or fatty acid when used singly, or when a smaller proportion of fatty acid was used in the ratio.

Further evidence in this regard was established by Dubos (39, 40), who reported marked growth stimulation of tubercle bacilli in the presence of inhibitory fatty acid following the addition of crystalline serum albumin to the culture medium. This outcome was more apparent in media treated with a quantity of organic acid just less than the amount known to effect inhibition, a finding analogous to the results in table 16 and 17. Moreover, Dubos also pointed out that disappearance of toxicity was concomitant with the clearing by albumin of the lipid-caused opalescence in the medium. He likened this clearing to the possible formation of a lipoprotein complex, and, whereas previously an increased acidity caused diminished growth, the effect of pH in the presence of this complex became negligible, a finding parallel to data recorded in table 16 and 17.

Such enhanced growth responses occurring at particular lipid:agent ratios suggested to both Oyama and Dubos that a nutritional deficiency had been filled. From the nature of agents tested and by token of results obtained, these authors, separately, claim the agent's role to be that of detoxicant

for a growth-essential quantity of lipid. The basis for this claim is well founded, and is adopted to explain the function of albumin and Tween 80 in this study.

To account for the above acknowledged detoxifying action by proteinaceous agents, especially that of bovine plasma albumin, redirects attention to the phenomenon of binding or complexing, typical generally of native protein, and not characteristic only of albumin. The affinity of proteins for ions varies widely (26), but that of serum albumin far surpasses the affinity of any other protein yet examined. This comparison pertains as well to anion binding by albumin, and in particular to fatty acid anions, which are more tightly bound as the carbon chain increases in length in the aliphatic series (26, 30). To help account for the unsuccessful substitution (with one exception) of other proteins as detoxicants of fatty acids (32, 41), is the fact that serum albumin can bind considerably more than one fatty acid anion per molecule (43); in fact will bind three to six molecules of oleic acid tightly enough to prevent bacteriostasis, or, when in equilibrium with free acid in a saturated, neutral solution, will bind a total of nine molecules (32). The single excepted protein is beta-lactoglobulin from milk. Klotz (26) rated the fatty acid anion binding power of this protein a poor second to that of serum albumin; while egg albumin is far less effective, and the serum globulins (Armour's fractions II, and III-1) have negligible fatty acid-binding powers. These findings corroborate initial data presented in this study, as do also the reports that serum albumin loses its power to bind fatty acid (8), or to act as detoxifying agent (32) following heat denaturation, or enzymatic hydrolysis.

Reversible binding of fatty acids also supports the claim that proteinaceous agents function as detoxicants. Reversible binding has been

substantiated for saturated fatty acids up to 12-carbon caprylate by use of C^{14}-labeled anions and serum albumin (34). The binding reversibility of 18-carbon oleic acid is based, however, on the above mentioned work of Davis and Dubos (32), in which the amount of albumin required to just extinguish opalescence of a saturated acid solution, showed nine molecules bound to albumin at equilibrium. As a result of this reversal capacity, microorganisms are enabled to obtain the needed quantity of fatty acid without accompanying bacteriostasis, since, as the minute quantity of free fatty acid is used up by the cells, more will be released by dissociation of the complex in an effort to maintain equilibrium.

To verify detoxifying action by the protein, serum albumin, one need only to recall its function in blood. For example, it exhibits affinity for anions of chemotherapeutic agents, and reduces the hemolytic action of fatty acids on erythocytes (30, 32), besides also fulfilling the role of transporting agent, and of solubilizing agent of water-insoluble compounds.

The fatty acid:serum albumin complex might also be compared with lipoprotein in the blood. Of the two blood lipoprotein fractions, the beta form is said to be 75 percent lipid, complexed with an unidentified protein. Besides, the function of these two blood fractions is akin to that of albumin, i.e., to render water soluble such lipids as cholesterol, carotene and the steroids (29). In fact, a very recent report (44) concerning pleuropneumonialike organisms, a rather fastidious group as a whole, pointed out that the growth factor in mammalian sera responsible for supporting optimal growth was a lipoprotein of which the protein portion could be effectively replaced by bovine serum albumin or beta-lactoglobulin, if used in larger amounts than that required of the original protein. Cholesterol, not

fatty acid, however, was the essential lipid portion of the growth factor.

It is also interesting to recall that Knaysi (45) claimed that the bacterial cytoplasmic membrane is constituted principally of a highly stable lipoprotein. Besides, control of permeability is one of the two known functions of this membrane. These two factors suggest an application of Gibbs' rule, as utilized (46) in the theory of diffusion of fatty acid through insect cuticle, which is a network of protein-lipoid interfaces. That is to say, after initial molecular orientation, diffusion of the oriented molecule along such interfaces (Gibbs' layer) may be extremely rapid, unless hindered by a preferential absorption, or stabilized because of a balance between opposing protein and lipoid phases (a factor related to ratio of protein:lipid). Diffusion may either be chiefly in the protein phase, or in the lipoid phase, depending on the nature of the diffusate. This concept, then, could feasibly be the bridge linking the lipoprotein constitution of the cytoplasmic membrane (whose function is permeability regulator), and the stimulus of the exterior lipid:agent ratio. This ratio relationship could hinder or promote nutrient diffusion and thereby determine whether growth was inhibited or stimulated.

Knaysi (47) also declared that the lipid content of the cytoplasmic membrane is usually low during the early stages of active growth, but that content increases as cells mature. In conjunction with this, it is fitting to mention that the usual lag phase, previous to growth stimulation of Lactobacillus casei upon either oleic or linoleic acid-containing media, completely disappears following addition of bovine serum albumin. Subsequent absence of the expected lag (seven days) in initial growth may well represent ready orientation at the cell wall of the fatty acid:albumin complex with the cell lipoprotein content (48). Likewise, in fitting

agreement with this observation of Knaysi's, is the belief that cell permeability changes with the age of the culture, as evidenced by the fact that cellular sensitivity to toxic substances is maximum during the active growth period (47).

Supporting the previously acknowledged detoxifying action by Tween 80 and other non-ionic detergents is their capacity for protective binding that has been verified in several reports. For example, Davis (32) showed that tubercle bacilli, though known to be sensitive to free oleic acid, even to the amount normally found in Tween 80, nevertheless were enabled to grow in the presence of small additions of this free fatty acid to media, if Tween 80, purified of its contaminating content of acid (38), was also present. Likewise, the data of Tomarelli et al.(42), previously mentioned, cf., provides further basis for such binding and also for detoxifying capabilities of these agents, as does also that of Williams et al. (49), who report that oleic acid, essential for growth of Lactobacillus bulgaricus, is non-toxic only within a very narrow range of concentrations. However, addition of Tween 40 or 60, themselves inactive as growth agents, render oleic acid non-toxic over a greatly expanded range of concentrations, and extend the pH range over which growth occurs. Likewise, adequate amounts of Tween 80, alone, because it is a water-soluble, esterified derivative of the growth-essential oleic acid, proved equally as stimulatory as Tween 40 plus oleic acid.

Such binding between fatty acid and Tween as these molecular interactions demonstrate, and upon which potential detoxifying action is based, is not surprising when recalling the large quantities of various lipids that are dispersed clearly in water solution by Tween.

When comparing the binding interactions manifested by albumin with those of Tween 80, the data tabulated in this study indicate that the latter apparently binds less effectively, over a less extensive area. And though less effective per unit concentration, Tween 80 presumably shows reversible binding of free fatty acid (32), just as does albumin.

Protection of red blood cells against hemolysis from added oleic acid is also afforded by Tween 80, but for only a short period, i.e.,30 minutes at 37°C., while hemolysis occurs if held overnight in the refrigerator (32).

The mechanism by which the non-ionic detergent compounds detoxify is not clear. Two important factors, however, are apparently interrelated; that of solvation of the unsaturated fatty acids by esterification, essentially a change in polarity, following which growth promotion is usually favorable; and a change in surface tension and subsequently cell membrane permeability. A sufficient change in surface tension presumably permits better contact with nutrients of the environment, as well as improves rate of diffusion, effects which are comparable in nature to those of lipoprotein at the cell wall.

8. The effect of crystalline bovine plasma albumin and Tween 80 as nutrients. Because the proposed role of detoxifying agent presupposes the function of albumin and Tween 80 to be protective rather than nutritive, demonstration of this premise was sought by incorporating each, singly, into minimal agar medium, that is, one lacking the constituent, tryptose. The amounts of each agent utilized (0.05 percent by weight of albumin, and 0.05 percent Tween 80, on a liquid basis) had previously on tryptose agar, supported normal colonial development and high colony count of strain 119.

The trial resulted in complete absence of growth, both on the control, mediumconsisting only of agar, salt, and dextrose, and on the minimal

Tween 80-containing agar; but an estimated count of 2000 colonies, none larger in diameter than 0.2 mm., and almost water-clear in appearance, developed on the minimal albumin-containing agar. Repetition confirmed this suboptimal colonial development.

Thus, the function of albumin is still open to conjecture. Formation of lipoprotein, and beneficial effect therefrom, is not improbable. This deduction is explained on the basis that lipid content is known in both crystalline albumin and in agar. Cohn (50, 29) has disclosed that native albumin resists crystallization if first defatted, and that from 0.05 - 1.0 moles of fatty acid per mole of albumin have been detected in the crystallized product. Perhaps it seems unlikely that such a quantity of lipid could affect growth. However, if extracted, "lipid-free" albumin is used in place of crystallized bovine plasma albumin in the chemically defined medium sufficing for cultivation of the Reiter treponeme, the medium is rendered absolutely growth ineffectual. Subsequent addition of the extracted lipid to the medium restored growth-promoting activity (41). So, too, the presence of an inhibitory agent in agar was reported for gonococci (51). Later, methanol extraction of agar and subsequent reincorporation of the extract in the medium, resulted in complete inhibition of Neisseria gonorrhoeae at a 0.5 microgram/ml. concentration, a quantity in agreement with the equivalent inhibitory concentrations of oleic and stearic acids (52). Thus, the extremely low concentrations at which fatty acids may act, favorably or otherwise, is more fully recognized. And by the same token, absence of growth on minimal Tween 80-containing agar can easily be the effect of excess free fatty acid accompanying the Tween, in addition to its non-protein nature.

9. The effect of starch, and activated carbon in media. The disclosures that addition of starch to solid media successfully reversed the inhibitory action of agar for gonococci (51), and, subsequently, that the inhibitory factor for gonococci in commercial agars was trace amounts of lipoid material (52), as well as the report (53) that the linear component of non-hydrolyzed starches was capable of adsorbing fatty acids reversibly, prompted the addition of starch to tryptose agar. The medium was made to contain 0.4 percent starch by the addition before autoclaving of the proper amount of an aqueous ten percent stock solution of soluble starch.

Similarly, it was mentioned in the literature that activated carbon, long known chemically as an adsorbent of fatty acids, effectively replaced blood, or blood serum requirements in media for such organisms as meningococci, gonococci, and Hemophilus pertussis (54, 55), all sensitive to fatty acids. Therefore, along with starch, the effect of activated carbon was tested in tryptose agar. A ten percent, autoclaved, aqueous stock suspension of Norite-A (activated) was used for preparation of 0.2 percent Norite-A-tryptose agar.

Growth results on both agar types closely resembled that obtained on the 0.05 percent Tween 80 agar, viz., a high colony count of normal size colonies.

A modification in the use of activated carbon, achieved by mixing an amount of Norite-A in the aqueous solution of dissolved tryptose and then removing the Norite-A by filtration, after which preparation of tryptose agar was carried out as usual, also resulted in the development of a high colony count of strain 119, but of colonies somewhat smaller in diameter.

The two methods of treating agar medium with activated carbon, showed in the first instance that growth was promoted in the presence of the adsorbent, and secondly, that the actual presence of activated carbon during

incubation was unnecessary for comparable growth results. The consequent conclusion, therefore, is that activated carbon, acting in an adsorptive capacity, removed a growth inhibitory agent present in tryptose, and although a strong adsorbing agent, did not simultaneously remove all growth-essential constituents, although comparatively, resultant colonial diameters were slightly smaller after the latter procedure.

Thus, by analogy, if there remains any doubt that the principal contribution of the albumin fraction of blood to growth promotion of the fastidious Br. abortus 119 is not nutritional (as had been tacitly assumed), but rather, protective, and that instead of lacking a nutrient, the medium contains a factor in excess, then the effect of the two agents, starch, and especially activated carbon because without value as a nutrient, should conclusively dismiss the uncertainty.

To confirm this analysis, a more sensitive means, that of evaluating growth promotion in broth media was inaugurated. The following data measure growth of a minimum number of bacterial cells, as well as focus attention on the tryptose ingredient, and preclude any question of agar purity.

The effect of activated carbon in broth media is recorded in table 19. For comparison, trypticase soy broth, though known to support growth of the fastidious 119 strain, was exposed to Norite-A in the same manner as was tryptose broth, as follows: 1. sterile Norite-A was added to sterile broth at the time of cell inoculation, 2. Norite-A was mixed with either tryptose or trypticase in aqueous solution, and removed by filtration before addition of the other ingredients and sterilization (as described above for agar), and 3. a combination of these two procedures. The 0.2 percent Norite-A, added at the time of inoculation, settled rapidly, filmwise, on the floor of the broth bottle and left a clear supernatant. Care

TABLE 19

THE EFFECT OF ACTIVATED CARBON (NORITE-A) ON GROWTH OF TWO CO_2-DEPENDENT STRAINS OF BR. ABORTUS IN BROTH

Mediums	Turbidity[a]			
	Strain 119[b]		Strain 3038[c]	
	48 hrs.	72 hrs.	48 hrs.	72 hrs.
1. Tryptose	-	-	<1+	<4+
2. " and Norite-A[e]	2+	5+	2+	5+
3. " (N.A.T.)[d]	2+	4+	2+	4+
4. " " and Norite-A[e]	3+	5+	3+	5+
5. Trypticase soy	1+	4+	3+	5+
6. " " and Norite-A[e]	4+	5+	3+	5+
7. " " (N.A.T.)[d]	2+	4+	3+	5+
8. " " " and Norite-A[e]	4+	5+	3+	5+

a = degree of growth by turbidity
b = inoculated with 20 cells
c = " " 10 "
d = Norite-A treated (1%), and Norite-A removed by filtration
e = 0.2 percent
- = no turbidity

was taken not to disturb the liquids at any time after inoculation. The effect of these media on two strains of Br. abortus is also contrasted in table 19.

The results affirm the outcome observed on agar, and point out that the contrast in the two Br. abortus strains is effectively minimized as soon as the medium is Norite-A treated -- initial and terminal growth of both strains is shown to be comparable. In fact, strain 3038 shows improved growth after Norite-A treatment of tryptose. Lastly discerned is the tendency for those bottles of medium that contained additional Norite-A to show more rapid growth. (Note trypticase soy broth).

Continuing the comparison of these two Br. abortus strains, the data in table 20 illustrate the effect on growth of three different concentrations of sodium oleate added to both tryptose and trypticase soy broth, each previously freed of any inhibitory agent by Norite-A treatment. The substitution of a known for an unknown inhibitory agent and subsequent comparison of the absence or presence of activated carbon during incubation, points out that Norite-A removed the added oleic acid as well as the unknown inhibitor (table 19), which strengthens the presumption that the latter is fatty acid.

The results also reveal that when the concentration of fatty acid is not growth inhibitory, the presence of added Norite-A may initiate earlier cell reproduction, but that when the fatty acid concentration is growth inhibitory, the added Norite-A acts like albumin and causes the reversal of inhibition, (see #6 and #12 of table 20). Furthermore, where reversal is noted, though the lag phase is longer, the rate of multiplication in the logarithmic growth phase must have been more rapid in order to show in 72 hours a terminal turbidity equivalent to that of other broths. Either result, earlier growth response, or reversal of inhibition

TABLE 20

THE EFFECT OF FATTY ACID ON GROWTH OF TWO CO_2-DEPENDENT STRAINS OF BR. ABORTUS IN NORITE-A-TREATED BROTH

	Na-Oleate Concentration Added to Medium (micro-gms./ml.)	Mediums	Turbidity[a] Strain 119[b] 48 hrs.	72 hrs.	Strain 3038[c] 48 hrs.	72 hrs.
1.	0.1	Tryptose (N.A.T.)[d]	1+	4+	2+	5+
2.	"	" " + n-a	2+	5+	3+	5+
3.	1.0	" "	1+	5+	1+	4+
4.	"	" " + n-a	2+	5+	2+	5+
5.	10.0	" "	-	-	-	3+
6.	"	" " + n-a	-	5+	-	5+
7.	0.1	Trypticase soy (N.A.T.)[d]	2+	5+	3+	5+
8.	"	" " " + n-a	2+	5+	3+	5+
9.	1.0	" " "	2+	5+	3+	5+
10.	"	" " " + n-a	2+	5+	2+	5+
11.	10.0	" " "	-	-	-	4+
12.	"	" " " + n-a	-	5+	-	5+

a = degree of growth by turbidity
b = inoculated with 20 cells
c = " " 10 "
d = Norite-A-treated (1%), and Norite-A removed by filtration
e = 0.2 percent
- = no turbidity
n-a = Norite-A, 0.2 percent

suggests the possibility that activated carbon too, may supply by reversible binding, nontoxic fatty acid as needed.

Use of albumin (table 21) in a manner comparable to that of Norite-A (table 20) reveals the similar effect of the two agents. The tabulated data summarize the results obtained for albumin and indicate that 0.005 percent was the lowest concentration of albumin enabling 19 cells to multiply in regular tryptose broth. Because 0.20 percent concentration of Norite-A initiated earlier cell reproduction, it must have exerted a more extensive adsorbent action than 0.005 percent albumin. In fact, this quantity of Norite-A is shown to have an action comparable to that of 0.012 percent albumin.

Addition of either a like concentration of albumin, or of Norite-A to both tryptose broth and Norite-A-treated tryptose broth containing sodium oleate, disclosed by comparison that the concentration of unknown inhibitor in regular tryptose broth, when expressed in terms of oleic acid, was slightly less than ten micrograms/ml.. Furthermore, there was verification of Dubos' observation, namely that subsequent clarification of broth attests addition of a sufficient quantity of albumin to protect against the antagonistic effect of sodium oleate. Other data not tabulated indicated that a fluctuation in inoculum size, totaling as few as ten cells, was sufficiently critical to affect reproducibility of results.

Lastly, the data in table 22 point out that inhibition in tryptose broth passes rapidly from reversible bacteriostasis to an irreversible bactericidal effect. Cell survival in trypose broth depends, therefore, on two factors, 1. concentration of protective, adsorbing agent; and 2. length of exposure to inhibitory agent. Thus it follows that the more dilute the protective agent, the more readily does a bacteriostatic inhibi-

TABLE 21

THE CONCENTRATION OF ALBUMIN NECESSARY TO OBTAIN GROWTH IN TRYPTOSE BROTH, AND IN NORITE-A-TREATED TRYPTOSE BROTH CONTAINING A TOXIC CONCENTRATION OF SODIUM OLEATE

Na-oleate Concentration Added to Medium (micro-gms./ml.)	Mediums	Albumin Concentration (percent)	Turbidity[a] Strain 119[b]	
			48 hrs.	72 hrs.
0	Tryptose	0	-	-
0	"	0.005	±	5+
0	" + n-a	0	2+	5+
10	Tryptose (N.A.T.)[d]	0.005 (Did not clear broth)	-	-
10	" "	0.012 (Cleared broth)	±	4+
10	" " + n-a	0	-	5+

a = degree of growth by turbidity
b = inoculated with 19 cells
d = Norite-A-treated (1%), and Norite-A removed by filtration
n-a = Norite-A, 0.2%
- = no turbidity

TABLE 22

THE EFFECT OF ALBUMIN CONCENTRATION ON GROWTH IN TRYPTOSE BROTH

Agent	Concentration of Protein (percent)	Turbidity[a,b]			
		48 hrs.	72 hrs.	126 hrs.	174 hrs.
Albumin	0.24	2+	5+		
"	0.1	2+	5+		
"	0.05	2+	5+		
"	0.005	±	5+		
"	0.0025	-	- (added albumin, 0.24%)	2+	5+
"	0.001	-	- (added albumin, 0.24%)	-	-
Whole bovine serum	0.4	1+	5+		
Control		-	-	-	-

a = degree of growth by turbidity
b = inoculated with 19 cells of Br. abortus strain 119
- = no turbidity

tion become bactericidal. On the other hand, albumin concentrations in excess of the protective amount just needed to promote cell multiplication, show no variation in rate of initial cell reproduction.

The results at the termination of the broth culture study parallel data obtained for colonial development on agar in so far as determining that the fastidious 119 strain is more sensitive to oleic acid than is either of the typical Br. abortus cultures. Yet, comparison of these two types of media, broth and agar (cf. table 13), discloses a toleration of a lesser concentration of sodium oleate in agar. This outcome is not to be discredited since in agar the effect of an inhibitory agent is more locally concentrated.

Because of the adsorbent capacity of both albumin and activated carbon, either agent may reverse the growth inhibitory effect of tryptose broth or agar. Thus, for growth of fatty acid sensitive bacteria, the important factor is not the amount of fatty acid present in media, but the extent and the firmness with which it is bound, or detoxified. Subsequently, concentration of adsorbing agents in media, as well as the time of their addition ultimately determine whether the inhibitor will be bactericidal, bacteriostatic, without effect, or perhaps even beneficial.

DISCUSSION

The following points established in this study concerning the action of albumin and of Tween 80 facilitate an explanation of some undiscussed results in the data.

Toxicity of a fatty acid for Br. abortus is a function of concentration, and varies with the bacterial strain. Albumin in media is beneficial primarily as a protective agent, because it has unsurpassed ability to attract and bind ions, particularly fatty acid anions. However, after heat denaturation or after enzymatic hydrolysis, this binding property characteristic of the native molecule is destroyed. Yet, because binding is reversible, such fatty acids are non-toxic and may act as nutrients. The extent of the latter effect is determined by the ratio of fatty acid content to that of albumin. Thus, should the number of fatty acid anions exceed the available number of albumin binding sites, the unbound anions, depending on concentration, would still exert growth inhibition. On the other hand, a ratio with a surplus of albumin molecules, would bind available fatty acid anions far more tightly, so that ease of reversibility would be greatly decreased, and fatty acid essentially non-available. Consequently an optimum ratio (neither of these extremes) would show optimum growth stimulation, and be characteristic for each bacterial species or strain. Furthermore, the composition of the optimum lipid: protein ratio may closely resemble the lipoprotein constituent of the bacterial cytoplasmic membrane, the role of which is to solubilize water insoluble nutrients and to control rate of diffusion of nutrients into

the cell, essentially a control of surface tension, as well as to control cell division. The greater the similarity in composition of these two constituents, the greater, theoretically, would be the ease of passage of nutrient into the cell and the better the growth stimulation.

Tween 80, by means of an emulsifying film, also acts as a protective agent. A molecular association with fatty acid, this film exhibits a degree of binding, though far weaker and attractive over a field of force far smaller than that characterizing albumin. Esterification provides a non-toxic form of fatty acid to the bacterial cell (39), but unless purified, Tween is contaminated with unesterified fatty acid, which to some extent is detoxified and made nutritively available by reversible binding. However, the extent of detoxification is determined by the ratio composition of Tween to unesterified free fatty acid, and the result is finally manifest in either growth stimulation or inhibition. Tween 80, a surface active agent able to reduce the surface tension of the medium, may exert further benefit as a surface tension depressant by improving the rate of nutrient diffusion into the cell, a function similar to that of lipoprotein in the cell membrane.

As the result of establishing the presence of a toxic, growth inhibitor (presumably fatty acid), in tryptose, and of recognizing the probable interaction between albumin and fatty acid anions, the pattern taken by the colony count in Tables 3 and 4 can be clarified. The highest colony count occurred at the optimum ratio of albumin to fatty acid. Here, the inhibitor ions are presumably adequately bound. At lower albumin concentrations, inhibitor ions exceed available albumin binding sites and so continue to exert a degree of inhibitory effect, indicated by decreased colony size and count. On the other hand, albumin concentrations exceeding

those in the optimum zone, perhaps bind inhibitor ions so tightly that colonial development may be hindered by this lessened availability. For example, maximum growth of the Reiter treponeme (41), required a higher concentration of oleic acid in the presence of albumin than in its absence. More significantly, however, albumin in excess of the optimum, probably acts to bind other nutrients as well as fatty acids, so that multiplying cells consequently must draw nourishment from a larger area of agar; as a result, colony count decreased.

Egg albumin autoclaved in the agar medium, also produced a colony count of characteristic pattern, although in comparison to crystalline bovine plasma albumin, its binding capabilities are slight. Thus, any detoxifying action is ascribed to micelle formation. Hence, increase in number of egg albumin molecules renders fatty acid inactive for growth inhibition. Yet, presence of an excess number of egg albumin molecules would counteract no more than would an equivalent number, nor would they impede availability of other nutrients. Consequently colony count in table 5 did not diminish in the presence of excess egg albumin.

In the data in table 12, the notation that colonies tended to cluster in the region of the pad inoculated with Tween 80 can be better understood by recalling the surface tension depressor nature of the water soluble Tween. Thus, both an increase in rate of diffusion of nutrients into the cell, as well as the faster diffusion of Tween 80 through the medium and subsequent increased ease of drawing nutrients in the direction of the pad can account for this result.

The protection afforded cells by the emulsifying film of Tween 80 is lacking in Span 80 because of its water insoluble nature. The contaminating oleic acid in Span, coupled with the inhibitor content in tryptose,

combine to exhaust the weak protective powers of killed cells in the concentration used in the medium, a protection also apparently the result of micelle aggregation, a colloidal engulfing. Beneficial growth promotion of Johne's bacillus by use of killed bacterial cells in medium has also been reported (56).

The apparent contradiction that Tween 80 proves a better growth-enhancing agent than albumin for the aerobic Br. abortus (Table 18) is not as inconsistent as it may at first seem. Tween 80, being better able to diffuse because of its detergent properties, can spread into the medium more quickly than albumin, and, too, can more quickly provide the cells with nutrients (including detoxified fatty acids), by virtue of its surface depressant action. Since, relatively speaking, strain #2308 tolerates a high concentration of free fatty acid, the fact that sodium oleate is more loosely bound when in conjunction with Tween than when complexed with albumin, may be an additional factor contributing to the broader enhancement zone exhibited by Tween.

Besides the effect of detergent properties, difference in the diffusion rate of the two agents is probably further influenced by the dynamic attractive powers of albumin. Thus, increase in molecule size by complexing, also may retard outward diffusion of albumin from the pad.

Comparison of breadth of enhancement zones promoted by both albumin and Tween 80 (cf. pp.34), reveals that those for colonial growth of Br. abortus 119 were wider than those of strain 2308. This result may be related to ratio of fatty acid to agent. Since the concentration of sodium oleate inhibitory for #119 is more dilute than the concentration inhibitory for #2308, there would be fewer bindable anions in the former medium and diffusion rate would consequently not be as promptly retarded as when fatty

acid anions were more numerous at a higher concentration.

That decrease in colony size at the rim of plates #2 and #3 (table 18) is not caused by sodium oleate alone, can be verified by comparison with plate #1. The reduction in colonial size in the peripheral area may result from the coincident presence of fatty acid not yet detoxified, and a dwindling supply of nutrients, a consequence of migration inward to the disc.

The results of this study point out how unpredictable is the sensitivity of three strains of Br. abortus to fatty acid. But strains within other bacterial species often show a similar varied response. For instance, four micrograms/ml. of free oleic acid inhibits a heavy inoculum of a virulent Hemophilus pertussis, whereas one non-virulent strain can grow in the presence of 160 micrograms/ml., and another is not inhibited by 400 micrograms/ml. of oleic acid (57). In another case (58), a penicillin-dependent strain of staphylococcus, suppressed by two micrograms/ml. of sodium oleate, and subsequently inhibited on regular stock laboratory medium except in large inocula, responded readily following addition of either whole blood, serum, 0.2 percent activated carbon, or penicillin to the medium. Ether-extracted medium and Norite-A-treated medium (followed by removal of Norite-A), likewise supported growth. Furthermore, growth inhibition due to addition of sodium oleate to ether-extracted medium, could be reversed with a suitable concentration of penicillin. Yet, the parent strain of this variant, not suppressed by even 1000 micrograms/ml. of oleic acid, was inhibited by penicillin.

Unlike any other bacterial growth-inhibitory agent, the effect of fatty acid may be reversed, and apparently there is but a small margin between the stimulatory and the inhibitory concentration. Yet these substances presumably are normal constituents in bacterial cells. In a study

of the lipid fraction (five to six percent of the dried cell weight) of one strain of Br. abortus (59), 51.5 percent of the more abundant phosphatid fraction was found to consist of fatty acids. The less plentiful acetone-soluble fat fraction, upon saponification, also yielded fatty acids, and so did the trace quantity of "bound" lipids in the cells.

Insignificantly small quantities of fatty acid may be growth-essential or growth-suppressing. The favorable influence of crystallized albumin on the cultivation of fastidious bacteria may be one related either to detoxification or to stimulation by supplying fatty acid in trace quantity, since such an amount is known to persist even after several recrystallizations. Blood or serum can likewise serve a similar dual purpose. But possible liberation of free fatty acids in growth-suppressing quantities by the action of lipase present in either blood, serum, or cells, may give rise to erratic growth results, depending on the idiosyncrasy of the bacterial strain. For this reason, the improvement noted in the successful isolation of Brucella from blood culture, by following the practice of heating blood to 56° C. for fifteen minutes, may not only be due to inactivating the bactericidal antibody-complement system (4), but also to the inactivation of the lipase content (57).

Similarly, despite the adsorbing action of starch, the erratic results that occur when cereal starches are incorporated in media, may be traced to their unpredictable content of fatty material. On the other hand, potato starch, naturally free of fat, has proved more successful in media and has long been used for the cultivation of both Brucella and H. pertussis.

Whether serving as a source of fatty acid or as a detoxifier, albumin is known to improve the successful isolation of a variety of fastidious microorganisms. Furthermore, it serves to replace a serum requirement in

several instances. This is true for Leptospira canicola, Plasmodium knowlesi, Trichomonas vaginalis, Treponema pallidum, several rickettsiae, and viral agents (41). Ultimately, however, whatever the fatty acid requirements of the organism, the amount of fatty acid present in media will apparently not be troublesome, as long as it is sufficiently detoxified, and is available if essential, yet without influence if inhibitory.

SUMMARY

A fastidious CO_2-dependent Br. abortus Type II (Wilson), the colonial development of which was known to be inhibited on tryptose agar unless killed brucella cells fortified the medium, produced normal colonial growth on agar fortified with blood serum. The serum fraction responsible for growth enhancement was found to be albumin. Heat denaturation and enzymatic hydrolysis diminished the growth-stimulatory property of albumin, while complexing albumin with sugars neither augmented nor diminished this property.

Tween 80, but not Span 80, produced a similar growth-enhancing effect upon this strain, as did also starch, and activated carbon.

It was concluded that the enhancing effect of albumin was one of protection, and not a nutritive action. Likewise, the effect of the other growth-stimulatory agents was also protective.

The inhibitory agent in the tryptose constituent of the medium was thought to be of a fatty acid nature.

Sodium oleate, (oleic acid), added to tryptose agar medium was inhibitory to the colonial development of the above mentioned strain of Br. abortus at the minimum concentration of 3.28×10^{-7} M, while for another strain, a similar inhibitory response did not occur until the concentration was increased to at least 3.28×10^{-3} M. Furthermore, oleic acid exerted a bacteriostatic effect that could be reversed, or become bactericidal. The outcome depended upon: 1. the bacterial strain, 2. the concentration of the fatty acid, 3. the number of cells in the inoculum,

4. the duration of cell exposure to oleic acid before addition of protective agent, and 5. the ratio of the concentration of fatty acid to the concentration of protective agent. In fact, a particular concentration ratio of oleic acid:albumin was more growth-stimulatory than was albumin alone.

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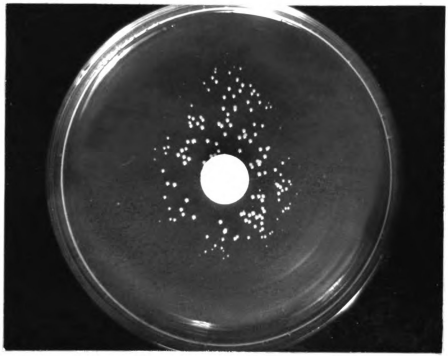

Plate 1. Zone of enhanced colonial growth of the fastidious Br. abortus 119 on tryptose agar effected by addition of 0.2 ml. of whole bovine serum to the paper disc. (72 hour incubation).

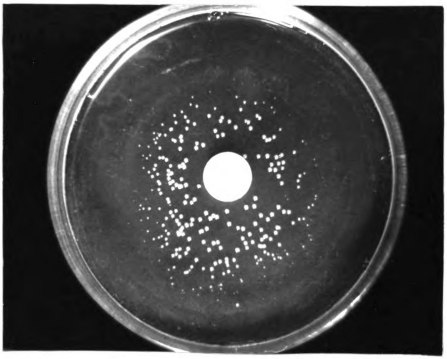

Plate 2. Zone of enhanced colonial growth of the fastidious Br. abortus 119 on tryptose agar effected by addition of 0.1 ml. of crystallized bovine plasma albumin (4.8 percent) to the paper disc. (72 hour incubation).

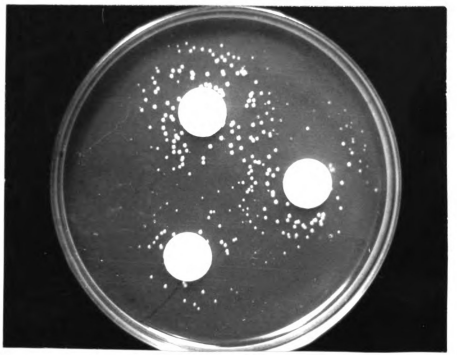

Plate 3. Effect of decreasing amounts of crystalline bovine plasma albumin (4.8 percent) to the paper disc on colonial development of Br. abortus 119. Passing clockwise from top to bottom, 0.1 ml., 0.05 ml., and 0.02 ml. (72 hour incubation).

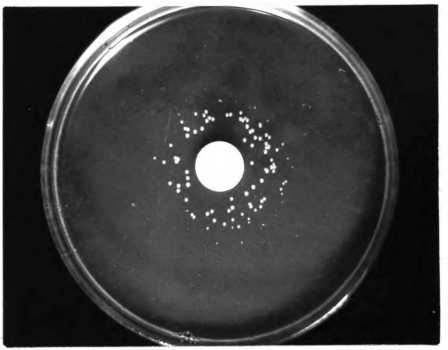

Plate 4. Diminished zone of enhanced colonial growth of Br. abortus 119 on tryptose agar following addition of 0.1 ml. heat-denatured (60°C. for 30 min.) crystallized bovine plasma albumin (4.8 percent) to paper disc. (72 hour incubation).

Date Due
