GROWTH REQUIREMENT OF STAPHYLOCOCCI[1]

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In the course of an investigation on the chemistry of bacterial metabolism it became desirable to develop a simple medium for the cultivation of staphylococci, free from the unidentified and complex compounds present in "extract," "infusion" and "hormone" media. This report describes an attempt to isolate chemically and to identify a substance found essential to "activate" such a medium, thus permitting the growth of staphylococci.

Attempts to develop a synthetic medium, suitable for the growth of all organisms have been made by Uschinsky (1893) and Doryland (1916). Mueller (1922) has studied in detail the chemical requirements for growth of streptococci.

As a metabolic base for our tests the medium of Uschinsky was used, as was also the casein hydrolysate utilized by Mueller. Preliminary tests showed that these two solutions were incapable of supporting growth of freshly isolated staphylococci for more than two generations. In practically all cases there was no growth in the first transfer, provided care was taken to keep the inoculum, and consequently the amount of original medium transferred, very small.

It is generally known that the addition of meat extract to a peptone base medium will permit heavy growth of many organisms, including the staphylococci. Tests showed that meat extract would, in a similar manner, "activate" synthetic media, permitting luxuriant growth of staphylococci. Meat extract alone, when dissolved in saline, proved an adequate medium only in concentration above 0.5 per cent. The presence of 0.01 per cent of extract, however, in the hydrolyzed casein solution, or in

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Uschinsky's medium promoted heavy growth. The following tests represent a study of some properties of this "activator."

CHEMICAL PROPERTIES

Effect of heat and reaction. Nine flasks containing 100 cc. of 2 per cent meat extract were prepared. In three flasks the reaction was adjusted to pH 7.0; to pH 5.0 in a second set of three; and to pH 9.0 in the remaining three. The flasks were heated in an autoclave at 20 pounds pressure for periods of twenty, forty and sixty minutes, as indicated in table 1. The results given in table 1 show the amount of growth resulting when 1 cc. of the extract solution was added to 5 cc. casein medium, inoculated with *Staphylococcus albus* and incubated. The results indicate

TABLE 1

pH	AMOUNT OF GROWTH		
	20 minutes in autoclave	40 minutes in autoclave	60 minutes in autoclave
5	Good	Good	Slight
7	Heavy	Good	Good
9	Slight	None	None

that the activator is heat stable at neutrality or in a slightly acid range; but is markedly decomposed by heat in an alkaline solution.

To test the possibility of an amino acid being concerned in activation a sample of meat extract was subjected to acid hydrolysis. Twenty grams of meat extract were boiled in 200 cc. of 10 per cent sulphuric acid for eighteen hours, under a reflux condenser. The sulphuric acid was removed with barium hydroxide, and the hydrolysate tested for activating power. This property had been entirely lost. Suitable control tests were made to eliminate the possibility of toxic salts of barium remaining in the medium. The probability of an amino acid acting as the activating substance was greatly decreased by this test.

Dialysis. To determine if a non-coagulable protein or other substance of large molecular size was concerned in the activation the ability of the active substance to dialyze was tested. A

collodion bag was cast in a 250 cc. Erlenmeyer flask. After washing and removal, 100 cc. of the 2 per cent meat extract solution was introduced into it, and allowed to dialyze for twenty-four hours against distilled water. The dialysate and the residual fluid were found to possess equal amounts of activator, demonstrating its ability to pass through the collodion membrane.

Solubility. The solubility of the active substance in the more common extractive reagents was tested as described below.

Ether. Five cubic centimeters of the 2 per cent extract solution were shaken with 10 cc. ether. On standing, a dark brown viscous layer separated out, as did an intermediate layer of gelatinous consistency. The upper ether layer was siphoned off, and the ether evaporated on a water bath. There was no residual material.

Alcohol. To a 5 cc. portion of 2 per cent extract solution 25 cc. of 95 per cent ethyl alcohol were added. The solution was heated to boiling, filtered, and the precipitate washed with alcohol. Only a very small amount of material was precipitated. The filtrate and washings were combined and evaporated to dryness on the water bath, and the residue taken up in 5 cc. water. Culture tests showed that the filtrate contained the activating substance in amount equal to the original solution, demonstrating its solubility in alcohol.

Benzene. Twenty-five cubic centimeters benzene were added to 5 cc. of the 2 per cent meat extract solution. After agitation the benzene layer was siphoned off, the benzene evaporated on the stream bath, the residue taken up in water and tested. No activating material was present in this residue.

Ethyl acetate. Five cubic centimeters meat extract solution were treated with 25 cc. ethyl acetate, and allowed to stand after shaking. The upper layer was removed, the ethyl acetate removed by evaporation on a water bath, the residue taken up in 5 cc. water, and tested. No active material had passed into the ethyl acetate layer.

Acetone. Five cubic centimeters of the extract solution were treated with 25 cc. acetone. A very bulky, brown precipitate resulted, which could easily be packed by centrifugation, and a clear yellow supernatant decanted off. The acetone was removed from this supernatant by evaporation and the residue taken up in 5 cc. water. Culture tests showed that the original amount of activating substance was contained in the acetone extract.

This test was repeated using 50 cc. extract solution and 500 cc. acetone. The brownish precipitate was washed twice in acetone, then suspended in water and heated until acetone-free. Tests showed it to be entirely free of activating substance, while the filtrate, treated as before, contained the full amount.

These tests show the activating material to be soluble in 80 per cent alcohol and in 90 per cent acetone. Since the bulk of inert material precipitated by acetone is much greater, and since the acetone is more easily removed owing to its greater volatility, use of this chemical seemed desirable for preliminary precipitation. An additional series of tests were made showing that the precipitation of inert material and the extraction of the active material were both equally effective in any concentration of acetone from two to ten volumes. Since further studies on the active substance could be made more easily on concentrated material, 1 pound of meat extract was dissolved in 1000 cc. water, and treated with 3000 cc. acetone. The precipitate was packed by centrifugation, the supernatant decanted, and the acetone-removed from it by heating on the steam bath.

Precipitation. To determine some general chemical properties of the active substance the following series of tests with reagents generally employed in the isolation of bases were made.

Barium hydroxide. Cold saturated barium hydroxide solution was added to 5 cc. of the acetone soluble fraction until precipitation was complete. The precipitate was washed with barium hydroxide, the washings and filtrate combined, made barium-free with sulphuric acid and concentrated on the water bath to 5 cc. Tests showed that all of the active substance was contained in the filtrate.

Silver-barium. To another 5 cc. portion of the acetone-soluble fraction 10 per cent silver nitrate was added until an excess was indicated by the barium hydroxide test. Saturated barium hydroxide solution was then added until the mixture was decidedly alkaline to litmus. The precipitate was filtered off and washed with dilute barium hydroxide. The filtrate and washings were combined and made barium-free with sulphuric acid, then the silver removed with hydrogen sulphide. The filtrate was heated on the steam bath to drive off excess hydrogen sulphide, and concentrated to 5 cc. Culture tests showed it to possess no activity.

A second lot of the acetone-soluble portion was similarly treated. In addition the washed barium-silver precipitate was suspended in water and treated with sulphuric acid and hydrogen sulphide. Culture tests showed that all of the active substance had been precipitated, and that the greater portion of it could be recovered from the precipitate.

Mercuric sulphate. A third 5 cc. portion was treated with an excess of 5 per cent mercuric sulphate dissolved in 5 per cent sulphuric acid. The precipitate was filtered off and washed with 5 per cent sulphuric acid. The filtrate and washings were combined and treated with hydrogen sulphide to remove the mercury. Results of culture tests showed that the active substance had not been precipitated.

Phosphotungstic acid. A fourth 5 cc. portion was treated with an excess of 30 per cent phosphotungstic acid in 5 per cent sulphuric acid. The precipitate was filtered off and washed with 5 per cent sulphuric acid. The filtrate and washings were combined and freed from phosphotungstic and sulphuric acids by the addition of finely powdered barium hydroxide, until the reaction was slightly alkaline, then excess barium was removed by adding sulphuric acid to slight acidity. The precipitates were removed and the filtrate concentrated to 5 cc. Culture tests showed the filtrate to be entirely inactive.

The test was repeated using 25 cc. of the acetone-soluble portion. In addition, the phosphotungstic acid precipitate was suspended in water and treated with barium hydroxide, similarly to the filtrate. All of the active substance had been precipitated and was recovered quantitatively from the precipitate.

These tests show that the active material can be precipitated by barium-silver or by phosphotungstic acid treatment. Since manipulation in the latter process was found to be easier, and also the possibility of loss of the active material by adsorption on metallic sulphides avoided, the remaining bulk of the acetone-soluble portion was treated with phosphotungstic acid and the active substance recovered from the precipitate.

Removal of inert material by crystallization. Preliminary tests showed that the remaining portion of the extract contained large amounts of amino acids and "muscle extractives." Since these substances are not extremely soluble and crystallize well, their removal by fractional crystallization was attempted.

Another pound of meat extract was treated with acetone and phosphotungstic acid as described, and combined with the remainder of the

first portion. This solution, slightly acid with sulphuric acid, was heated on the water bath. After considerable concentration a crop of crystals separated out, resembling in appearance half-melted snow, but yellowish in color. These were removed by filtering on a Buchner filter, and recrystallized from water. The second recrystallization yielded colorless crystals. The "mother liquors" were combined and added to the original "mother liquor," and further concentrated. In all, seven such crops of crystals were obtained. Each crop was tested separately and each was found to be free of activating substance after the second recrystallization.

No extensive attempt was made to identify the substances that crystallized out. Carnosine was recognized by its reaction with copper carbonate. Reduction of picric acid indicated the presence of creatinine. Reaction with nitrous acid demonstrated the presence of the amino group, probably contained in dibasic amino acids.

Further concentration. An appreciable bulk of the remaining inert material was removed by treatment with the methods described below.

Mercuric chloride. Preliminary tests showed that an appreciable amount of inactive material could be removed by the use of mercuric chloride, without precipitation of the active substance. Consequently cold, saturated mercuric chloride was added to the remaining solution until precipitation was complete. The precipitate was filtered off, and washed well in mercuric chloride solution. The filtrate was acidified with sulphuric acid and hydrogen sulphide passed through it until free from mercury. The mercuric sulphide was filtered off, the solution neutralized with sodium hydroxide and evaporated to dryness on the water bath and taken up in alcohol, and the alcohol insoluble inorganic salts present were removed by filtration. The solution was again dried on the water bath to remove alcohol and taken up in water. A considerable loss of activating substance occurred during this manipulation, probably due to loss through adsorption on the mercuric sulphide.

Picric acid. Tests made at this point showed appreciable amounts of amino nitrogen to be present. Since this probably represented dibasic amino acids, their removal with picric acid was attempted. Preliminary tests showed that a considerable portion of the remaining material could be precipitated by addition of picric acid, and that this material was inert. To the remaining solution cold saturated picric acid solution

was added in excess. A heavy precipitate formed, taking crystalline form on standing over night on ice. This was filtered off and washed with picric acid. The filtrate and washings were combined and made slightly acid with hydrochloric acid, and the picric acid removed by extraction with ether. Tests showed the active substance to be uninjured.

Additional tests. The original amount of meat extract had now been concentrated to a mass of 265 mgm. This material was taken up in 100 cc. of water. This concentrated bulk made practical tests with some rarer chemical reagents.

Gold chloride. To 5 cc. of the concentrate, 10 per cent gold chloride was added in excess. A slight precipitate formed, which was filtered off after standing over night. The precipitate was washed in dilute hydrochloric acid, suspended in water, and treated with hydrogen sulphide until free of gold. The filtrate was similarly rendered free of gold. The volume of each was adjusted to 5 cc. and tests for activating power were made. Neither the filtrate nor precipitate showed any activating property, probably due to loss of the activating substance through adsorption on gold sulphide.

Platinic chloride. To another 5 cc. portion of the concentrate, 5 per cent platinic chloride in 50 per cent alcohol was added. No precipitation resulted, but on the addition of 95 per cent alcohol a fine, granular precipitate formed. This was filtered off, washed in 95 per cent alcohol, dried and taken up in water, then the platinum removed by treatment with hydrogen sulphide. Platinum was removed from the filtrate in a similar manner. On testing, no active material could be demonstrated either in the filtrate or precipitate. Here again, the loss was probably due to adsorption.

Picrolonic acid. To a third 5 cc. portion of the concentrate a saturated solution of picrolonic acid in 95 per cent alcohol was added. A precipitate formed, but was found to consist only of picrolonic acid itself, rendered insoluble by the lowered alcohol concentration.

Electrodialysis. A dialysis cell was built, with parchment membranes approximately 1 inch apart, and adjustable in number up to twelve. Carbon electrodes were used. Dialysis was attempted with varying currents and times, in solutions of high and low conductivity, and of different ranges of reaction. The uniform result was that the activating substance completely disappeared. A possible explanation is that the substance became concentrated in a cell having an alkaline

reaction, where, in its more purified and consequently more unprotected form, it was rapidly decomposed.

Nitrogen content. Micro-Kjeldahl nitrogen determinations indicated 17.3 per cent nitrogen. It was observed that the solution would not clear in a digestion mixture containing copper as a catalyst, but cleared rapidly on the addition of hydrogen peroxide. On treatment with nitrous acid no nitrogen was produced. With methyl iodide a characteristic pyridine reaction was obtained. Free pyridine was absent since there was no precipitation with potassium ferrocyanide.

Activating power. Tests for activating property were made, with the results shown in table 2.

TABLE 2

1	5 cc. casein alone		Sterile
2	5 cc. casein plus 0.1	mgm. concentrate	Heavy
3	5 cc. casein plus 0.01	mgm. concentrate	Heavy
4	5 cc. casein plus 0.001	mgm. concentrate	Good
5	5 cc. casein plus 0.0001	mgm. concentrate	Good
6	5 cc. casein plus 0.00001	mgm. concentrate	Fair
7	5 cc. casein plus 0.000001	mgm. concentrate	Trace
8	5 cc. casein plus 0.0000001	mgm. concentrate	Sterile

Using tube 5 as the limit of activity, computation shows that 1 mgm. of the concentrate should be sufficient to activate 50 liters of a synthetic medium.

FORMATION BY BACTERIA

Since certain organisms, most notably the tubercle and colon bacilli, are known to possess the ability to grow profusely in synthetic media, the possibility of their producing a substance with the property of activating synthetic media suggests itself. Borrel, Boez and Coulton (1923) have observed such a substance in media in which tubercle bacilli have been grown.

Two liters of hydrolyzed casein were inoculated with *B. coli-communior* and incubated for four days, the acidity being adjusted twice daily during this period. This procedure yielded a heavy growth of bacilli, which were collected by centrifuging, and washed three times in

saline. The supernatant medium was filtered through a Berkefeld "V" filter, and concentrated to 100 cc. on the water bath. Culture tests showed that the medium contained no substance capable of stimulating growth of staphylococci.

The moist, washed bacilli were extracted for five hours with 70 per cent alcohol at 90°, under a reflux condenser. The suspension was filtered, and the filtrate evaporated to dryness on a water bath, then taken up in water. When this bacterial extract was added to hydrolyzed casein, to Uschinsky's medium, or even to the casein hydrolysate used for the growth of the organisms extracted, the medium so treated produced luxuriant growth of staphylococci after inoculation and incubation.

This test showed that these organisms, possessing the ability to grow well in synthetic media, have also the property of synthesizing a substance adequate to stimulate the growth of other species of bacteria. This phenomenon may well explain their ability to grow in a synthetic medium.

The above test was repeated, using ordinary "extract broth" inoculated with staphylococci. The extract of washed staphylococci did not possess the property of stimulating growth of staphylococci in hydrolyzed casein.

To determine whether the substance produced by the colon bacillus was similar in its chemical properties to that occurring in meat extract, the chemical tests done on the active substance in meat extract were repeated, using bacterial extract.

Ten liters of hydrolyzed casein were inoculated with *B. coli* and incubated as before, and the recovered organisms treated in the same manner. The extract was dried, taken up in water, precipitated with acetone, and the acetone-soluble portion treated with phosphotungstic acid, then concentrated by removal of inert material with mercuric chloride and picric acid. The active material from the colon bacilli reacted with all reagents, in a manner identical with that occurring in meat extract.

DISCUSSION

The chemical properties of the active substance contained in meat extract, capable of stimulating the growth of staphylococci,

place it in the general chemical class of "natural bases," which class also includes the substances effective in mammalian metabolism, designated collectively as "Vitamine B." These two substances appear to have identical chemical properties as far as they have been determined, as will be evident from examination of the chemical studies of Funk (1922) on the yeast vitamine. Indeed, the property of stimulating bacterial growth has been attributed to "Vitamine B" by Lloyd (1916, 1917), Shiba (1923), Shwartzman (1924–5), and Izrailskii and Runov (1928). Similarly, the ability to produce growth-stimulating vitamines has been claimed for certain bacteria by Pacini and Russel (1918), Sheunert and Schieblich (1923), and by Damon (1924). It is worthy of note that all the organisms for which this ability has been claimed belong in the group growing well on synthetic media.

These results are not considered as indicating a chemical identity between the vitamines promoting mammalian growth and those promoting bacterial growth; however, these two substances appear to belong in the same class of chemical compounds, and to possess in common the property of biological activity in extremely high dilution.

The data secured in these experiments suggest a possible classification of bacteria into three metabolic groups: (1) Organisms possessing the ability to synthesize an activating substance, adequate for themselves and certain other organisms, and by virtue of this property possessing the ability to grow in synthetic media (example, *B. coli*). (2) Organisms requiring an accessory substance in addition to basic energy-producing media, but unable to synthesize it, and growing well in synthetic media if traces of such an accessory substance be supplied (example, staphylococci). (3) Organisms requiring specialized substances for growth, in addition to activators and energy producing substances (example, *B. influenzae* requiring hemoglobin and an additional activator-like substance).

CONCLUSIONS

1. The substance present in meat extract capable of stimulating the growth of staphylococci belongs to the chemical group of

"natural bases." It is heat stable in slightly acid or neutral solutions, but rapidly destroyed in alkaline solution. It dialyzes through collodion membranes.

2. This activating substance is soluble in water, alcohol and acetone, but insoluble in ether, ethyl acetate or benzene. It is precipitated by phosphotungstic acid and silver-barium, but is not precipitated by silver nitrate, mercuric chloride, acidic solution of mercuric sulphate, picric or picrolonic acids. It is apparently strongly adsorbed on metallic sulphides.

3. The activating substance can be concentrated to a degree where less than 1 mgm. is sufficient to activate 50 liters synthetic medium, thus permitting good growth of staphylococci.

4. In chemical and growth promoting properties this substance present in meat extract behaves similarly to a substance produced intracellularly by *B. coli-communior*, an organism that possesses the property of growing profusely in synthetic media. Such a substance is not produced by staphylococci, which lack the ability to grow on synthetic media.

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