REQUIREMENTS OF A LANCEFIELD GROUP A STREPTOCOCCUS FOR GROWTH AND NEPHROTOXIN PRODUCTION

BY

Wilma Ethel Barshaw

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Bacteriology and Immunology,
McGill University,
Montreal, Quebec.

August 22, 1956

ACKNOWLEDGEMENTS

The advice and suggestions of Professors R.W. Reed and G.G. Kalz of McGill University, are gratefully acknowledged.

TABLE OF CONTENTS

INTRODUCTION

That all elements which are part of living organisms must be present in their environment, if they shall be in a position to propogate, is axiomatic. However the form in which these elements are useful for the building of cells, differs widely for various organisms. Simple non-pathogens can obtain all the nitrogenous compounds they require by synthesis from ammonia. The increasing complexity of nutrient requirements often associated with increasing pathogenicity, calls for the provision of more complex nutrients (McILWAIN et al. 1939 (a)).

It may be stated that any substance which an organism cannot synthesize and yet requires for its development, constitutes an essential nutrient for that organism. It occurs not infrequently that the development of an organism, while not strictly dependent upon the availability of some particular substance, can be greatly furthered by its presence. This type of compound has been referred to as a growth-stimulant (Van Niel, 1944).

The Lancefield Group A beta hemolytic streptococci are parasitic, highly pathogenic, and among the most complex species of heterotrophic bacteria with respect to their nutritional requirements. Parasitism, in general terms according to Fildes, (1934) is a result of a loss by organisms, of enzymes necessary

to synthesize cell material. When this occurs, these same organisms are no longer capable of growth in simple synthetic media, but require as well, several compounds supplied by the activity of other organisms for use as structural units, or for metabolic processes. It is for this reason that, growth requirements not being a matter of concern, such organisms were and still are for the most part, cultivated on empirical mixtures containing complex organic matter of animal origin, like peptone, meat extract, serum or egg albumin (Mueller, 1922 (a)).

The determination of the indispensable nutritional requirements for any particular micro-organism may be approached in one of two ways. On the one hand, identification could be made of one or more components of an undefined medium, be they either a source of nitrogen or some particular grouping found to play a part in supporting growth, by the addition of a growth stimulating complex to an otherwise deficient medium, This addendum could then be fractioned until its effect could be attributed to a definite compound or compounds within the complex. On the other hand, the problem could be attacked by analyzing the basic factors supplied by the physiological mixtures which are known to induce growth. Once any growth stimulating substance has been identified, it can be determined whether it acts as a building stone in supplying some necessary grouping in the

synthesis of either the bacterical cell per se or one of its by-products, or whether it simply initiates or accelerates some vital process. This information is of utmost importance when it is desired to characterize any substance elaborated by an organism (Bernheimer and Rodbart, 1948).

From the time pathogenic organisms were isolated in medical bacteriology by Loeffler in 1881, with the object of establishing their relationship to disease, proteins, peptones, meat extracts, and the like were used to enrich the culture media without any attention being paid to the specific growth requirementsof the organisms in question (Stephenson, 1948).

The advantages of a chemically-defined synthetic medium for biochemical study were appreciated early in the history of bacteriology. With a medium, the chemical composition of which is known, there is a much greater possibility of determining by exact study, the chemical nature of the products elaborated as a consequence of bacterial growth. This was an impossibility in a medium which contained complex undefined organic substances.

HISTORICAL REVIEW

By 1918, many synthetic media had been devised, most of them containing several inorganic salts, and having glucose or glycerol as the source of carbon, and organic ammonium salts, asparagin, or glycocoll as the nitrogen source. Among the most important of these earlier media were those of Cohn, Frankel and Uschinsky. The chief difficulty with these, however, was that the fastidious organisms of the pathogenic class did not find proper nutriment in such simple media (Robinson and Rettger, 1918).

Efforts to identify the exact nitrogen requirements of the Group A streptococci were for a long time interferred with, by the necessity of meat, liver, or yeast extract in the media as a source of the unknown growth requirements. It was not until all such substances were identified and available in pure form, that the extracts could be eliminated and the nitrogen requirements of these organisms be worked out in more exact terms.

The first study of the nitrogen requirements of pathogens was reported in 1911 by Bainbridge, who pointed out that certain bacteria required the presence of proteins as such, as well as inorganic salts and glucose in the culture medium.

In agreement with this, was a belief which Mueller (1921) held for a time; that the growth stimulating substance for the hemolytic streptococci which he obtained from certain

proteins was a definite constituent of the protein molecule. However, after isolating the active substance from a trypsin digest of casein and after treatment of this substance as one would purify a protein, he found it to be inactive as a growth stimulant. The failure of a trypsin digest of purified casein to support growth while that of impure casein was satisfactory, confirmed the need of the hemolytic streptococci for a non-protein substance, and further pointed out the ability of these organisms to utilize a nitrogen source midway in complexity between the intact protein and inorganic nitrogen.

The possible presence in organic complexes of unknown substances, quite apart from nitrogenous material, and thought to be of biological importance in nutrition, was also a suggestion of other early investigators.

Freedman and Funk in 1922 (b) stated that cases in which it had been definitely shown that bacterial growth was due to the presence of protein alone, and not to any impurities it may have contained were extremely rare. In most instances it was possible to show that other growth factors were present as added constituents or as adhering impurities.

Mueller in 1920, reported that while a peptone free beef heart infusion broth would support an abundant growth of hemolytic streptococci, short boiling with charcoal removed this property entirely. The addition of commercial peptone, or of a sulphuric acid hydrolyzate of certain proteins, such

as casein, reactivated the charcoal treated infusion, and heavy cultures of streptococci were obtained on such a mixture, while neither alone provided the slightest trace of growth.

From the evidence he obtained, Mueller (1922 (a)) concluded that two clases of organic compounds in addition to carbohydrates were required for the growth of Group A streptococci; the first was supplied by protein degradation products, like casein hydrolyzate, the second by extractives of meat. Both occured together in impure proteins and ordinary meat infusion, and in all probability each class was made up of several different factors.

Mueller (1922 (b)) then carried out a separation of these two classes of substances with the hope of elucidating the components of each. The active non protein material in peptones, protein-hydrolyzates and meat extracts, was precipitated by means of mercuric sulphate. This precipitate was further subdivided into two fractions, active only when mixed together, by treatment with silver sulphate and baryta ($Ba(OH)_2$). The silver sulphate precipitate contained histidine in addition to the active substance. This fraction escaped precipitation by phosphotungstic acid under certain conditions, but was readily destroyed by this reagent. The silver sulphate filtrate was not precipitated by phosphotungstic acid, and contained a considerable quantity of a sulphur containing amino acid.

Using the same Group A hemolytic streptococcus as

did Mueller, Freedman and Funk (1922 (a)), attempted to shed more light on the identity of the substances growth-promoting for these organisms. They too reported the presence in beef heart infusion and peptone, of certain non protein substances which showed a strong growth-stimulating activity for hemolytic streptococci. These active substances could be extracted from their natural sources by shaking with certain adsorbents, such as fuller's earth and norite charcoal, and could be recovered by extracting the adsorbents with baryta, ($Ba(OH_2)$. The properties of these substances showed them to be of a vitamin-like nature; they were similar to if not identical with the vitamin D described by Funk and Dubin in 1920. They reported as well, the presence in beef heart infusion of another substance which was necessary for the growth of hemolytic bacteria, and this substance was thought to be associated with hemoglobin.

Hosoya and Kuroya in 1923, obtained similar results. They reported that Group A streptococci required something accompanying vitamin B1 in an alcoholic extract of rice bran. According to Hutner (1938), this factor was stable to heat and acid, destroyed by drastic alkali treatment, adsorbed by fuller's earth, and precipitated by phosphotungstic acid.

The relationship between the growth-stimulating substances which are adsorbed to protein, and vitamin D, were studied by Freedman and Funk, (1922 (b)). From hydrolyzates of what they termed purified casein, and commercial gelatin,

they obtained certain substances which showed a marked growth stimulating activity for hemolytic streptococci grown in beef-heart infusion previously treated with norite charcoal. Hydrolyzed purified egg albumin and lactalbumin showed only traces of such activity, whereas similar preparations of several other proteins examined, were inactive. These active substances were found not to be part of the protein molecule, and the amount of them present in the protein depended upon the physical and adsorptive properties of the latter, and the method and degree of its purification. The properties of these substances were examined, and all evidence indicated that they were probably related to if not identical with the water soluble vitamins - at that time collectively called vitamin B.

In this connection Funk and Dubin in 1921, reported that both vitamin B and D were removed from autolyzed yeast by fuller's earth.

In 1938 Kane and Subbarrow reported that their attempts to substitute a relatively simple chemically defined medium for the usual complex medium in the cultivation of hemolytic streptococci, indicated the need for essential accessory growth factors. Whether multiple or of a complex nature they had up until 1938 been unidentified. Preliminary experiments by these workers demonstrated the presence of these indispensable factors in liver extract. A medium consisting of the substance listed in Table 1, was incapable of inducing growth of a Group A streptococcus, but upon addition of a liver extract, growth equivalent to that obtained in meat infusion broth resulted.

TABLE 1

Glucose
Inorganic salts
Gelatin hydrolyzate
Tyrosine
Cystine
Glutamic acid
Tryptophane
Methionine
Valine
+

Glutathione ⟵ either ⟶ Aqueous liver extract
Thiochrome
Flavin
Nicotinic acid
Betaine
Glucosamine
+
Ca alcoholic precipitate of liver
Uracil
Guanylic acid
Xanthine
Hypoxanthine
Nicotinamide
Unidentified liver fraction

By fractionation, glutathione, thiochrome, nicotinic acid, betaine, flavin, and glucosamine were isolated from the liver extract. These substances were, however, active only with an additional fraction of a liver extract, the complete identity of which was undetermined.

Subbarrow and Rane in 1939 using the same basal medium as previously reported, supplemented with the substances known to be present in liver, and the calcium alcoholic precipitate of a liver extract, reported that the further addition of an unidentified fraction of liver extract, nicotinic acid amide in place of nicotinic acid, uracil, guanylic acid, xanthine, and hypoxanthine, resulted in a significant increase in the amount of growth of a Group A streptococcus.

Certain similarities between the properties of the calcium-alcoholic precipitate used above and those of pantothenic acid described by Williams and co-workers in 1939, suggested the possibility of their substitution. It was found that the amount of growth obtained with a purified preparation of pantothenic acid was equal to that obtained using the calcium alcoholic precipitate of liver extract.

In 1939 (a), McIlwain, Fildes, Gladstone, and Knight, reported that in the course of analyzing the nutrient requirements of a Group A hemolytic streptococcus it was found that growth did not take place in the medium consisting of the substances listed in Table 2, until an extract of meat was added.

TABLE 2

Glucose
Inorganic salts
Peptone
Cystine
Alanine
Thiamine
Pimelic Acid
Nicotinamide
Riboflavin
Uracil
Cytosine
Thymine
Guanine
+

Meat extract ⟵ either ⟶ Glutamine

Good growth was then obtained in some 16 hours, whereas in the culture without meat extract, no growth was visible during a period of 9 days. In order to elucidate the active principle in meat, these workers chemically fractionated fresh horsemeat, - first extracting it with sodium sulphate, and then precipitating it with mercuric acetate in neutral 60 per cent alcoholic solution. The mercury-precipitated material was then dissolved, and reprecipitated with phosphotungstic acid. The final product was an active concentrate of

meat, capable of supporting growth. That the active material in the meat extract may have been glutathione was considered, in view of the report of Kane and Subbarrow in 1938. However, owing to the fact that its properties were dissimilar to those of glutathione, and in the absence of demonstrable sulfhydryl groups, this idea was abandoned. Since it occasionally happens that an increase in the concentration of particular amino acids might have a critical effect upon growth, an experiment was carried out in which increased amounts of the constituent amino acids of glutathione - that is glycine, glutamic acid and cystine, were added separately to an otherwise complete medium. Unlike glycine and cystine, a high concentration of synthetic glutamic acid was capable of inducing growth, comparable in rapidity and mass with that resulting from the meat extract. However, qualitative and quantitative differences between the meat concentrate and synthetic glutamic acid led to the conclusion that glutamic acid could not have been the active substance in the meat extract. That glutamic acid may have been specifically associated with the active substance, in the sense that the organism could synthesize this substance itself, in the presence of a high concentration of glutamic acid, was a possibility which was investigated. Thus glutamine, being a derivative of glutamic acid and having properties similar to those of the active substance, was selected for testing. It was found that glutamine when added to the inadequate basal

medium in a low concentration, caused maximum growth of this Group A streptococcus in as little as 16 hours. Thus glutamine was required by this organism for growth. However, among the Group A streptococci the growth response to this substance was reported, by Snell (1951), to be irregular, and it is therefore considered that glutamine is concerned primarily with the initiation of growth.

In 1939 (b), McIlwain and co-workers also showed that no growth was evident when the peptone in the medium in Table 2, was replaced by acid hydrolyzed casein. They indicated that the necessary substance in the peptone which was absent from hydrolyzed casein, was probably pantothenic acid; and in fact, pantothenic acid concentrates in high dilution caused growth of their Group A streptococcus, in a medium where hydrolyzed casein replaced the peptone preparation.

Woolley and Waismann in 1939 demonstrated that acid hydrolysis of pantothenic acid which occurred when impure casein was treated with acid, resulted in a split of the amide linkage, leaving substantially intact the dihydroxyvaleric acid. The failure of the Group A streptococci to grow in a medium otherwise complete but containing the by-products of this hydrolysis in place of pantothenic acid itself, pointed out the inability of these organisms to form this amide linkage. According to McIlwain (1939 (b)) this could be compared with the limited ability of these same organisms to utilize glutamic

acid in place of glutamine, although they obviously form the amide linkages of their own proteins. It should be noted, however, that the amide linkage in both glutamine and pantothenic acid is unusual in not being in the alpha position to either an amino or a carboxylic acid grouping, but in glutamine it is in the gamma position and in pantothenic acid the beta position.

Woolley and Hutchings, again in 1939, using a medium which consisted of bactotryptone, liver extract, salts and glucose, obtained good growth of a Group A streptococcus. If, however, this medium was treated with alkali, its ability to support growth was destroyed. This ability was not regained moreover, by addition to the medium of the known alkali - labile growth factors, - riboflavin, pantothenic acid, and cozymase, until sodium sulphide and reduced iron had been added. This is shown in Table 3.

TABLE 3

Glucose Inorganic salts Tryptone	⟶	After alkali treatment required Riboflavin Pantothenic acid Cozymase (DPN) Sodium sulphide Reduced iron

The two latter substances acted as a reducing system. In fact no reducing substance was found to be specific; vitamin C, glutathione, and thioglycollic acid were equally effective. These results were in harmony with an hypothesis which was put forward

at that time, - that alkali - labile reducing substances were present in the liver extract as well as tryptone.

McIlwain, in 1940, used the medium found in Table 4, to study the growth requirements of the Group A streptococci. This medium alone was insufficient to support growth. Nor did the addition of a small amount of arginine to the medium have any stimulatory effect.

However, when a yeast preparation was used as supplementation, growth followed. As a result of earlier investigations, pantothenic acid was known to be required by these organisms, and when added in pure form in place of the yeast preparation, the medium was complete. If the casein hydrolyzate was substituted by the mixture of amino acids listed in Table 4A, growth was not supported.

TABLE 4

Hydrolyzed casein

Glucose

Inorganic salts

Alanine

Methionine

Cystine

Cysteine

Glutamine

Aneurin (Thiamine)

Nicotinamide

Riboflavin

Pimelic acid

Uracil

Thymine

Guanine

+

either

↙ ↘

Yeast extract | Pure pantothenic acid

TABLE 4A

Replaced by

Aspartic acid

Valine

Leucine

Alanine

Glutamic acid

Isoleucine

Phenylalanine

Lysine

Glycine

Proline

Hydroxyproline

Tyrosine

Arginine

Histidine

Hydrolyzed egg albumin

+

either

↙ ↘

Vitamin B_6 | Filtrate from a lead-mercury precipitate of yeast extract.

Even upon further addition of hydrolyzed egg albumin, little or no growth followed. However, when a filtrate from a lead-mercury precipitate of a hot extract of yeast was added, growth occurred. McIlwain then attempted to isolate and identify the substance required for growth of the Group A streptococci present in the hot extract of yeast. This substance was found to be adsorbed on norite charcoal, and when its properties had been investigated a parallelism was found between this factor and vitamin B_6 and actually vitamin B_6 could replace this yeast extract. Shortly after this discovery vitamin B_6 was described as a growth factor for streptococci by Hutchings and Woolley (1939). In the presence of vitamin B_6, substances such as serine, threonine, betaine, glucosamine, and inositol, appeared only to increase growth, they themselves having no effect in the absence of it. Urea, guanidine and creatine were without effect in the medium used in this experiment. McIlwain (1940), further stated that when using a medium which contained an impure preparation such as hydrolyzed egg albumin or casein hydrolyzate, it was not possible to obtain results indicating an all or none effect as regards the necessity for any one growth-promoting substance by a particular organism; this is so on account of the presence of suboptimal quantities of these or similar substances in the impure preparation being used.

On the basis of his experimental evidence, McIlwain pointed out several facts of importance concerning the

nutritional requirements of the Group A streptococci. (1) He stated that the effect of certain substances required for growth of the Group A streptococci, such as vitamin B_6 and pantothenic acid, remained equally important in the presence of excess of other growth requirements of the organisms, and could be replaced, if at all, by compounds very closely related in chemical structure. This was not true of substances such as glutathione, for example, whose effect could be reproduced by vitamin C. Still other compounds such as glucosamine, inositol, and certain amino acids, represented a type of substance whose individual effect in a medium was only definitely seen in the presence of suboptimal amounts of other factors, in this case vitamin B_6. McIlwain admitted that these different types of growth requirements were not clearly defined, but were categories somewhat arbitrarily made in discussing the nutritional requirements of any organism, which could vary from absolute dependence upon a material, to complete indifference to another. Also, conditions of growth could change the effect of a given compound from one class to another. For example, the requirement of the Group A streptococci for glutamine was found to be much stricter in the presence of a small inoculum, than in the presence of a large one, when it was replaceable by glutamic acid; and in any case, if the media contained traces of glutamine insufficient to allow growth, glutamic acid which was normally inactive, appeared to act as a growth factor.

(2) The lack of growth of a Group A streptococcus following the omission of a single amino acid from a medium, could not be taken to mean that this was an essential amino acid, stated McIlwain, since in another such medium where a different group of amino acids was used, growth may have occurred in the absence of this same amino acid, - the implication being, that such a substance although not essential, was involved in the metabolism of the organism and was derived by intermediate metabolism from other amino acids.

(3) It also came to the attention of this worker that in a case where glutathione was replaced by its constituent amino acids, growth did not occur in an otherwise complete medium. When, however, an increased amount of cystine or cysteine was present, growth appeared in from two to four days. Increased amounts of glutamic acid or glycine - the other two components of glutathione, had no effect or a deleterious one. Other sulfhydryl compounds were then investigated; mercapto-acetate, mercapto-succinate, and homocysteine, were found equally effective in replacing glutathione, the requirement for which obviously being of a non specific nature and indeed it could be replaced by either reduced iron and sodium sulphide, or by vitamin C.

Pappenheimer and Hottle in 1940, reported that they obtained a luxuriant growth of a Group A streptococcus, on a medium which consisted of inorganic salts, glucose, a hydrolyzed

gelatin preparation, and the factors listed in Table 5. The requirement of each of these factors for growth, was determined by the omission of each in turn from an otherwise complete medium.

TABLE 5

Glucose

Inorganic salts

Hydrolyzed gelatin

Glutamine

Tyrosine

Tryptophane

Uracil

Adenylic acid

CO_2 atmosphere air

Thioglycollic acid

Glutathione

Thiamine

Nicotinic acid

Pantothenic acid

Riboflavin

Vitamin B_6

Biotin

Uracil, while not essential, appeared to increase growth slightly. In the complete medium the limiting factor seemed to be glucose. Addition of more glucose, increased

growth proportionately until sufficient acid was produced to kill the organisms. The need for pantothenic acid and riboflavin agreed with the results of other workers. The necessity for glutamine confirmed the work of McIlwain. In agreement with him as well, was the finding that glutathione was not essential, for in this study a relatively high concentration of thioglycollic acid in the medium had the same effect. The finding with respect to vitamin B_6, that is, its requirement, also seemed to confirm the work of McIlwain. Thiamine was not previously reported as being essential for the growth of Group A streptococci. Although impure biotin was used, it seemed probable that it was necessary for growth. Subsequently a definite requirement of the Group A streptococci for pure biotin was reported by Hottle and co-workers (1941).

It was further suggested by Pappenheimer et al. (1940) that another essential factor or factors may have been present as impurities in the gelatin or in the glutamine, both of which were obtained from natural sources. This seemed unlikely in view of the luxuriant growth obtained, nevertheless, the possibility could not be overlooked. The requirement for adenine or related compounds by this strain was of interest. If the purine was omitted from the complete medium, no growth occurred within 40 hours. However, addition of adenine permitted growth to occur. Adenine was replacable by adenosine, or adenylic acid, by guanine, guanosine, or guanylic acid, and by

xanthine or hypoxanthine. It could not be replaced by uric acid or by the pyrimidines such as uracil or cytosine. Another interesting fact was that in the presence of 5 per cent CO_2 in the atmosphere above the culture, growth was greatly accelerated, and the surprising observation was made that even when purine was absent under these conditions, growth occurred. Further examination of the effect of CO_2 was made in detail. It was noted that (1) maximal growth when adenylic acid was present occurred within 20 hours, provided the CO_2 tension was 4mm or greater; (2) no significant growth occurred even after 40 hours incubation in the absence of adenylic acid when the CO_2 tension was below 2mm; (3) even when the CO_2 tension was high, a small but consistent increase in growth was apparent at the 40th hour if adenylic acid had been included in the medium. Another observation in this connection, was that the bicarbonate ion could not replace CO_2 in the absence of a purine. However, in the presence of both adenylic acid and bicarbonate, any slight growth of the organisms liberated sufficient CO_2 through action of the acid produced, to accelerate growth. This report of the accelerating effect of CO_2 was in harmony with those of other workers, and indeed McIlwain grew his cultures in 5 per cent CO_2. Whether CO_2 was necessary for purine synthesis or whether the purines play a role in the production of CO_2 by the organisms, could not be concluded from these experiments.

In 1941, Bass, Berkman, and Saunders, studied the

nutritional requirements of the Group A streptococci. These workers used the basal medium listed in Table 6.

TABLE 6

Hydrolyzed Eastman gelatin

Cystine

Methionine

Tyrosine

Phenylalanine

Lysine

Histidine

Threonine

Tryptophane

Inorganic salts

Nicotinic acid

Alanine

Hemin

Inositol

Cocarboxylase

Riboflavin

However, growth did not occur in this basal medium. Nor was growth supported when one or all of the substances listed in Table 6A were added to the basal medium.

TABLE 6A

Glutamine

Ascorbic acid

Pantothenic acid

Vitamin B_6

Coenzyme I

Catalase

Although all the known water soluble vitamins were included in this medium, in view of the lack of growth, it was apparent that there remained one or more unknown factors necessary for the growth of these organisms.

The need for pantothenic acid, glutamine and vitamin B_6 was, however, not excluded by these workers, inasmuch as these substances may have been present in trace amounts in the basal medium or in the fractions which were being tested.

An alcohol water extract of yeast was found to be the best source of the factor required for growth of the organisms used in this study. Extracts of liver and spleen were also active in this respect. (Substances in Table 6A were not included when these extracts were added).

In 1941, Woolley and Hutchings reported a study of the factors essential for the growth of certain hemolytic streptococci. They were able to cultivate organisms of Lancefield's Groups B and D on a medium which contained an acid hydrolyzate of casein, glucose, and inorganic salts, as

well as those substances found to be of nutrient value for other organisms. These are listed in Table 7.

TABLE 7

Glucose

Inorganic salts

Hydrolyzed casein

Tryptophane

Glutamine

Riboflavin

Vitamin B_6

Na pantothenate

Thiamine

Nicotinic acid

Biotin

Inositol

Choline chloride

Pimelic acid

Na thioglycollate

Adenine

Uracil

Aqueous liver extract

On the other hand organisms belonging to Lancefield's Group A, failed to multiply on such a medium without the further addition of an unidentified growth-promoting substance. The fact that a deficiency of a known amino acid was not involved was shown by the failure of an increased amount of casein hydrolyzate in the medium to stimulate growth. The unidentified

substance used in this experiment, occurred in liver as a water soluble, alcohol insoluble, non-dialyzable material. Its properties differed in many respects from those described for other unidentified growth-promoting substances.

Bernheimer and Pappenheimer in 1942, studied the factors required for massive growth of several Group A streptococci. They used the medium of Pappenheimer and Hottle shown in Table 5, and included the following important changes.

1 - An increase in glucose concentrations of from 3 to 4 per cent, i.e. from 2.5 gm. per litre to 3 gm. per litre.

2 - Frequent neutralization of the lactic acid formed.

In this connection Mueller and Klise in 1932 reported that glucose appeared to be essential in a medium used for the cultivation of hemolytic streptococci.

Friedman in 1939, showed that hemolytic streptococci formed lactic acid almost quantitatively from glucose.

It seemed obvious that by a splitting of this substance to lactic acid, the energy required for the growth of these organisms was made available. The amount of growth obtained was roughly proportional to the per cent concentration (W/V) of sugar in the medium up to the point where sufficient acid was produced to check the growth of the organisms. The presence of a buffer in the medium, alone did not permit

massive growth. However, the occasional addition of sterile sodium hydroxide solution to the medium during growth, kept the pH at from 7.2 to 7.8 and resulted in a considerably greater yield of organisms (Mueller and Klise, 1932).

The other changes made by Bernheimer and Pappenheimer (1942) were:

3 - A reduction in the total salt concentration to a minimum.

4 - An increase in the glutamine concentration from 50 - 200 mgm. per litre.

With these changes, growth equivalent to 250 mgm. streptococcal nitrogen per litre was obtained with most Group A strains as compared to 20 - 30 mgm. streptococcal nitrogen per litre obtained previously. These so called improvements, however, had an opposite effect on three of the Group A strains tested; they failed to grow. Two of these grew on Woolley's medium shown in Table 7. Aside from differences in the concentration of the substances in the two media, the only material actually absent from Woolley's medium and present in this one, was sodium bicarbonate. The failure of these strains to grow was unexplainable.

In 1942, Grossowicz described the properties of what appeared to be a new growth factor for Group A streptococci, since it supported a heavy growth of these organisms in an otherwise incomplete medium. The basal medium which he employed consisted of an acid hydrolyzate of casein, glucose

and inorganic salts, but it did not support growth. Any of the other substances listed in Table 8, added singly or in combination to the basal medium, failed to initiate growth.

TABLE 8

Hydrolyzed casein

Inorganic salts

Glucose

Nicotinic acid

Thiamine

Riboflavin

Ascorbic acid

Alanine

Thioglycollic acid

p - Aminobenzoic acid

Glutamine

Adenine

Uracil

However, upon addition of a small amount of tomato juice (filtered, sterilized and neutralized) to the basal medium, growth followed. He reported that the active fraction in the tomato juice was similar to the growth-promoting substance which Woolley found in an aqueous liver extract.

In 1944, Sprince and Woolley reported that the new growth factor for GroupA streptococci, discovered by Woolley in

1941, and whose existence was confirmed by Grosswicz in 1942, was distinct from any of the known vitamins. This substance was found to be growth promoting for organisms other than the Group A streptococci. For example, C. diphtheriae, Lactobacillus arabinosus, the propionic acid bacteria, and in fact even organisms of Lancefield's Group D were found to have a much shorter lag period if this substance was incorporated into the culture medium. The occurrence of this growth factor in partially hydrolyzed casein puzzled these workers, in view of the fact that unhydrolyzed or fully hydrolyzed casein was inactive. Strepogenin was the descriptive name given to this active substance and the term is used to denote a growth factor whose presence is required for the multiplication of the Group A streptococci.

Woolley in 1946 (a), commented on the general belief that the nutritional or growth-promoting powers of proteins were reflected entirely by their content of the various amino acids. The fact that casein caused more rapid growth in an experimental animal than did a mixture of amino acids, had been explained by the hypothesis that the nutritional superiority of some proteins was due to the presence in them of a specific factor, which appeared to be correlated with the occurrence of strepogenin. This substance had been found as an integral part of certain highly purified proteins, from which it was liberated by tryptic digestion. Indeed, proteins

proved to be by far the richest sources of the growth factor. This active agent was destroyed by hydrolysis with strong acid or alkali. After studying the various properties of this substance, this investigator concluded that strepogenin was a peptide or peptide-like substance. Woolley later in 1946 (b), stated that he had aquired considerable evidence which indicated that strepogenin was a peptide of glutamic acid. For Lactobacillus casei, derivatives of glutamic acid such as glutamine and glutathione also had activity. Serylglycyl-glutamic acid was synthesized and tested for strepogenin activity. This tripeptide displayed only partial activity of strepogenin for L. casei, and it is possible that it is a fragment or relative of strepogenin.

Woolley in 1948 enhanced the evidence which existed concerning the peptide or peptide-like nature of strepogenin, the active material in the partial hydrolyzate of proteins, liver extract, and tomato juice. Although several peptides of glutamic acid possessed strepogenin potency, some specificity of action in relation to structure could be discerned. Thus although glycylalanyl-glutamic acid was shown to possess true strepogenin activity, the addition of just one more amino acid residue to the chain as in glycylalanylleucyl-glutamic acid, rendered the compound inactive. Thus the structure of the glutamic acid peptide was of importance in determining its strepogenin activity.

In 1955, Woolley et al. altered his previous statement concerning the structure of the substances which displayed strepogenin activity. That some specificity of structure was involved before a peptide exhibits high strepogenin activity, could not be overlooked. From the activity of many glutamic acid containing peptides, it may have been argued that for strepogenin activity, only a glutamic acid peptide would be required. Such an argument would have been negated however, by the inactivity of the glutamic acid peptide-isoleucyl glutaminyl-asparagine. Similarily a case could have been made for cysteine-containing peptides, which would likewise have been defeated by the inactivity of the disulphate of CySH-Pro-Leuc-Glyc (NH_2). Indeed, there seemed to be little justification, for stating that the only requirement for strepogenin activity was a peptide containing any one particular amino acid.

Again in 1955, Woolley and co-workers enhanced the existing evidence indicating that the active material in a partial hydrolyzate of protein, like casein hydrolyzate, was a peptide or a group of related peptides, Synthetic oxytocin was found to display high strepogenin activity for Lactobacillus casei. Likewise, arginine-vasopressin also possessed considerable strepogenin activity.

The free peptide derivative of oxytocin that is, the heptapeptide amide disulphide derivative whose formula is the disulphide of L-isoleucyl - L - Glutaminyl - L - asparaginyl - L - cysteinyl - L - prolyl - L - leucyl - glycinamide had a

strepogenin potency much the same as oxytocin itself. However, it showed negligible avian-depresser and oxytocic activities. The authors did however, mention that before these results could be applied to the Group A hemolytic streptococci, further studies would have to be done, since it is known that this group has a more exacting strepogenin requirement than does L. casei.

Wilson, in 1945 (a), using the medium listed in Table 9, in which casein hydrolyzate was the only undefinable substance, found that Group A streptococci failed to grow.

TABLE 9.

Glucose

Inorganic salts

Casein hydrolyzate

Cystine

Tryptophane

Glutamine

Asparagine

Thioglycollic acid

Choline Chloride

Biotin

Nicotinic acid

Pyridoxine

Ca Pantothenate

Thiamine

Riboflavin

Continued Table 9

Adenine sulphate

Xanthine

Uracil

If however 10 per cent rabbit serum was added to the medium as a supplement, the organisms multiplied. Moreover, certain strains of Group A streptococci which grew well in this medium, failed to grow at all when horse serum was substituted for the rabbit serum. Other strains grew well whichever serum was used. Thus approximately one third of the Group A streptococci required a factor in rabbit serum, which could not be supplied by horse serum. This factor according to Wilson (1945, b), could be replaced by yeast nucleic acid, and certain of its derivatives, such as xanthine, guanine, and hypoxanthine. Certain other derivatives such as adenine and uracil were inactive. These nitrogenous bases were considered to serve as precursors of nucleic acids. In the organisms in which these bases were non-essential, vitamins were known to play a part in their systhesis.(Snell 1951), Stephenson in 1948, reported that these bases were important growth stimulants for certain Group A streptococci, but added that they appeared to replace one another in the case of this group of organisms.

Ivanovics and Euler reported in 1950, that in a medium containing glucose, inorganic salts and a casein hydrolyzate, growth was obtained of a Group B streptococcus.

However, it was not until the other substances listed in Table 9A were added that growth of a Group A strain was obtainable.

TABLE 9A

Glucose

Inorganic salts

Casein hydrolyzate

Tryptophane

Glutamine

Thioglycollic acid

Biotin

Nicotinamide

Pyridoxine

Ca pantothenate

Thiamine

Folic acid

They reported too that other strains of Group A streptococci required an unknown growth factor or factors for growth. Neither choline nor sodium linoleate had any stimulatory effect. It should be noted that according to Slade (1954), no hemolytic streptococci have been found which require p-aminobenzoic acid. Nevertheless, folic acid which contains this compound is required by certain members of Group A. It is probable that such strains are unable to incorporate p-aminobenzoic acid into the folic acid molecule (Snell, 1951).

Slade and Knox in 1950, reported the construction of a so-called defined culture medium which enhanced the formation of streptolysin O by a Group A streptococcus. The medium developed consisted of the substances listed in Table 10.

TABLE 10

Trypsin hydrolyzed casein

Glucose

Inorganic salts

Nicotinic acid

Pyridoxal

Pyridoxine

Thiamine

Riboflavin

P-Aminobenzoic Acid

Biotin

Folic acid

Asparagine

Choline chloride

Inositol

Guanine

Xanthine

Uracil

They reported that the minimal vitamin requirements of the type 3 organism used in this study, were supplied by nicotinic acid, riboflavin and pantothenate. There was a

marked stimulation of lysin formation, however, in the presence of thiamine. This may have been due to a general improvement in the nutritional status of the cells, which resulted in a quantitative increase per cell of the enzymes required for lysin systhesis. Thus the presence of thiamine may have resulted in the production of cells that were more efficient in the formation of streptolysin O.

Todd in 1938, reported the presence of streptolysin O, in broth filtrates of Group A streptococci. This lysin was elaborated by the cells in the absence of a reducing agent, but its presence was evident only upon the addition of such a substance.

Slade and Knox (1950), on the other hand, reported that the formation of streptolysin O, was dependent upon the presence of an added reducing agent in the medium at the start, whereas optimum growth occurred in the presence or absence of such a compound. The fact that growth of this Type 3 strain occurred without concomitant production of the hemolysin indicated according to these workers, that the enzymatic systhesis of the lysin required a reducing potential of a certain level. This possibility was supported by the fact that increasing quantities of the reducing agent beyond the optimal level, caused a decrease in the amount of lysin formed, although a heavy growth of the organisms was obtained. Whether the enzymes required for lysin systhesis were elaborated during growth and remained inactive

in the absence of a reducing agent, or whether these enzymes did not arise at all in the absence of a reducing agent, was not determined. .Slade and Knox, further stated that the possibility of cystine, glutathione, sulphite or thioglycollate serving as a source of sulfhydryl groups for the protein of the lysin, was unlikely in view of the fact that ascorbic acid could serve equally well in this system. A threefold increase in glucose concentration would not yield the proper potential for lysin formation. Cysteine hydrochloride was the most active of all such agents tested and was required in a concentration below that normally used when it was an essential amino acid for a particular organism. These results suggested that an optimum reducing potential was required for maximum lysin production. In this connection, Bernheimer (1949), reported that 0.1 per cent cysteine abolished the inhibitory effect of shaking on the formation of streptolysin S by resting cells. This may be another example of the requirement for reducing agents in lysin formation.

Slade in 1951, reported that when the pancreatic digest of casein in the medium listed in Table 10, was substituted by a mixture of 19 amino acids,many strains of Group A streptococci grew only after an extensive lag period. The lag period could be eliminated by the addition of a nondialyzable preparation obtained from the pancreatic digest of vitamin-free casein, and whose properties indicated the presence of complex peptides.

Acid hydrolysis of the mixture yielded approximately 10 dialyzable and ninhydrin positive substances.

Slade, Knox and Slamp, in 1951, investigated the amino acid requirements of a Group A streptococcus using the same basal medium as found in Table 10, with the addition of sodium ethyl oxaloacetate, sodium fumarate, ascorbic acid and vitamin B_{12}, and again eliminating the casein hydrolyzate. They found the amino acids listed in Table 10A to be essential for growth under their experimental conditions. They pointed out that the amino acids required for growth of this Group A streptococcus, depended considerably upon the level of biotin, folic acid and vitamin B_6 in the medium.

TABLE 10A

Arginine

Cystine

Glycine

Histidine

Isoleucine

Leucine

Lysine

Methionine

Phenylalanine

Proline

Serine

Threonine

Tryptophane

Tyrosine

Valine

The amino acid requirements of this same organism were determined also, in the presence of a growth promoting peptide material, (prepared from hydrolyzed casein, by dialysis, freezing, and lyophilization). The presence of this substance in the medium did not eliminate the need for any of the essential amino acids listed above. These results indicated that the added peptide material did not serve as a source of free amino acids. However, the possibility of enzymatic hydrolysis of the peptides cannot be disregarded. If such a process did occur in the present experiments, isoleucine and valine were not made available in a form which could be utilized by the organisms for growth. (These two amino acids are known to be liberated upon acid hydrolysis of the peptide preparation). Also it seemed doubtful whether complete hydrolysis of the small amount of this substance present in a relatively large amount of medium, would liberate a sufficient quantity of any one amino acid to produce the response in growth which was obtained. Thus it appeared likely that the peptides contained in the preparation were utilized directly for growth.

The interest of these same workers (Slade, Knox, and Slamp, 1951) in glutathione, led to an investigation to determine whether this peptide would replace any of the amino acids required for growth. It was found that only cystine or cysteine could be replaced by glutathione. This activity of glutathione indicated that the metabolism of cysteine occurs at least in

part by way of the tripeptide or a common intermediate, since glutathione was unable to replace glycine or glutamic acid. Hanes et al (1950) and Fruton (1950), have suggested that the synthesis of cell protein may take place with the participation of glutathione or other gamma glutamyl peptides. The necessity of glutamine for the synthesis of such glutamyl peptides represented a reaction by which glutamine could be utilized by Group A streptococci. McIlwain, in 1946, pointed out that during glycolysis, the latter organisms quantitatively converted glutamine to glutamic acid and ammonia.

Slade and Slamp, in 1955, using the basal medium shown in Table 11, reported that crystalline ovalbumin containing free sulfhydryl groups (SH) fulfilled the requirements for growth of several Group A streptococci from small inocula, in this otherwise incomplete basal medium. The effect of strepogenin was nil for the first 18 hours, after which growth began and terminated at about the 24th hour.

TABLE 11

Glucose

Alanine

Cystine

Glycine

Histidine

Isoleucine

Leucine

Lysine

Methionine

Continued Table 11

Proline

Serine

Threonine

Tryptophane

Tryosine

Valine

Disodium Phosphate

Monopotassium Phosphate

Magnesium Sulphate

Ferrous Sulphate

Manganese Chloride

Zinc Sulphate

Adenine

Uracil

Glutamine

Nicotinic acid

Pyridoxal

Pantothenate

Thiamine

Riboflavin

They further showed that the presence of carbon 14, labelled ovalbumin in the synthetic medium resulted in a distribution of the isotope between the streptococcal cells and the culture fluid. The ovalbumin had to be treated previously either by shaking, irradiation, or heat in order to denature

the protein and thus unmask the sulfhydryl groups. Untreated ovalbumin was inactive, and the activity of that which had been heated was removed by oxidation. Other purified proteins were tested in both the heated and unheated states; crystalline preparations of pepsin, insulin, trypsin, chymotrypsin and ACTH, produced no significant growth response in the medium. Crystalline bovine serum albumin produced a 20 per cent response (as compared to ovalbumin), in 17 hours, which did not increase upon extended incubation. Crystalline vitamin B_{12} and coenzyme A were inactive. Neither was growth obtained in the presence of any of the following crude peptides.

(1) Anserine

(2) Carnosine

(3) Salmine

(4) DL-Alanylalanine

(5) DL-Alanylglycine

(6) Glycylalanine

(7) Glycylglycine

(8) Glycylglycylglycine

(9) Glycyl-L-Leucine

(10) Glycyl-L-Tryptophane

(11) Glycyl-L-Tyrosine

(12) Glycylvaline

(13) L-Leucyl-L-Tyrosine

(14) DL-Leucylglycine

(15) DL-Leucylglycylglycine

In conclusion it can be stated that by applying the knowledge which has accumulated from the nutritional studies of the Lancefield Group A streptococci, it is now possible to culture these organisms on a medium whose composition is almost entirely defined. This should greatly facilitate the isolation of the biologically active nephrotoxin in the culture filtrates of certain Group A streptococci, in a state approaching purity, and in sufficient yield to permit chemical studies. However, the mere fact that a toxigenic bacterial species can grow under a particular set of conditions, gives no assurance that it will produce toxin under the same set of circumstances. In fact, there does not seem to be any way of predicting whether the formation of a particular toxin will occur automatically as a consequence of growth.

EXPERIMENTAL PROCEDURE AND RESULTS

Experimental Strain:

The organism used in this study (strain #35) was kindly supplied by Dr. R.W. Reed of the Department of Bacteriology and Immunology of McGill University. It belongs to Lancefield's Group A and is a Griffith's Type 12 beta hemolytic streptococcus, isolated during an outbreak of acute nephritis in a rural area of Nova Scotia between October 1951 and January 1952. The extremely high carrier rate of this single Griffith's Type amongst a group of children showing 33 per cent incidence of acute nephritis, was considered by Reed (1953) as being highly suggestive of the origin of the outbreak. Because of this finding and since optimum yields of the nephritogenic substance were desired, this organism was employed for the present study.

The strain was received in the lyophilized state and was resuspended in 5 ml. of sterile "Pneumo-Broth" (see Appendix I Part A (1) from which a second tube containing 10 ml. of this broth and a blood-agar plate were inoculated; these were incubated at 37°C. for 24 hours. After this period the surface of the blood-agar plate revealed a pure culture, the colonies of which were in the smooth phase and were surrounded by a zone of hemolysis typical of beta-hemolytic streptococci. Microscopic examination of the organisms grown in "Pneumo-Broth", showed Gram-positive cocci in chains numbering on the average from five to six organisms.

A stock culture of this strain was maintained on a

blood-agar plate. Once a week it was subcultured onto a fresh blood-plate and following 24 hours incubation was stored in the refrigerator at 5°C.

1. The Comparison of Two Methods Available for Estimating the Relative Number of Organisms in a Bacterial Population.

Introduction:

Since this study involves the comparison of various media with respect to their growth supporting abilities a method of estimating the number of organisms obtained in such media was required.

Several methods of estimating the number of organisms in any bacterial population exist. Two of these are:

(A) Plate Count Method which provides an approximation of the number of living organisms in a bacterial culture at any one time.

(B) Measurement of the density of a culture as an estimation of the total amount of bacterial growth from the time of inoculation.

Materials and Methods:

(A) Plate Count Method:

From the stock blood-agar plate, 10 ml. of "Pneumo-Broth" was inoculated with the Type 12 organism and incubated for six hours at 37°C., after which a microscopic examination of the culture was made to verify its purity. To 8 ml. of sterile "Pneumo-Broth" 2 ml. of this six hour culture was added. At the same time a blood-agar plate was inoculated with 2 drops of this same culture. Both broth and plate were incubated at

37°C. for 18 hours. At the end of this time the plate was examined for contaminating colonies. Three sterile glass beads were added to the broth culture which was then shaken for one minute in order to disrupt the clumps of organisms and thus render the suspension homogeneous. Halving dilutions ranging from 1: 40,000 to 1: 1,280,000 of the shaken contents of the tube were made in 10 ml. volumes in "Pheumo-Broth". These particular dilutions were employed on the basis of a preliminary testing which indicated that such a dilution range would provide the number of organisms most easily counted on a blood-agar plate.

Sterile dropping pipettes calibrated to deliver 0.0125 ml. per drop were used, one per dilution. Each of the six blood-agar plates which had previously been incubated at 37°C. for three hours, with the lid removed slightly in order to remove excess moisture, was inoculated with four drops from one of the six dilutions prepared as above. The drops were spaced on the plates equidistant from each other and from the centre. The plates were rotated gently to ensure the spreading of each drop over approximately one-quarter of the surface area, thus encouraging the appearance of isolated colonies, but at the same time maintaining the boundary between drops. All plates were incubated for 24 hours at 37°C., after which the number of colonies per drop was counted with the aid of a magnifying glass.

Results:

These are presented in Table 13.

TABLE 13

Dilution	Average No. Colonies per Drop	Average No. Colonies per Drop Expected	x Per Cent Cumulative Error Dev.
$\frac{1}{40,000}$	35.2	-	-
$\frac{1}{80,000}$	16.6	17.10	- 2.90
$\frac{1}{160,000}$	12.1	8.55	+ 41.0
$\frac{1}{320,000}$	3.1	4.27	-27.0
$\frac{1}{640,000}$	1.2	2.03	-39.0
$\frac{1}{1,280,000}$	0.5	1.01	-50.0

x This figure was derived in the following way:

The difference between the expected and actual number of colonies obtained was divided by the expected number of colonies and multiplied by 100.

For example in the 1: 80,000 dilution the following calculation was made.

$$\frac{17.10-16.6}{17.10} \times 100 = -2.90 \text{ per cent.}$$

Conclusions:

The Plate Count method of determing the number of live bacteria in a Type 12 population was proven to be statistically inaccurate. When dealing with the lower dilutions, and thus with a larger number of organisms, the theoretical and actual values fall within a much closer range. However, in the case of the higher dilutions, these two values vary over such a wide range, that the large "Per Cent Cumulative Error Deviation" which results indicates that this is a somewhat inaccurate method of determining the relative number of living organisms in a population of the test organism.

(B) - Density of a population as measured in the Evelyn Photo-electric Colorimeter. (See appendix II Part B)

Materials and Methods:

From the stock blood-agar plate, 10 ml. of "Pneumo-Broth" was inoculated with the Type 12 organism and incubated for six hours at 37°C. At the end of this time the growth was examined microscopically for purity of the culture. Two ml. of this actively growing six hour culture was then placed in a flask containing 30 ml. "Pneumo-Broth" which had previously been rendered sterile and free from any solid material by passage through a number 5 membrane filter pad. (See appendix II Part C). The flask also contained 7 sterile glass beads and was fitted with a rubber stopper. It was incubated at 37°C. for 18 hours. A blood-agar plate was also inoculated with a drop of the six

hour culture and following incubation for 24 hours at 37°C., it was examined for contaminating colonies. After 18 hours incubation the tightly stoppered flask was shaken for one minute in order to break up the chains of bacteria and thus resuspend the sediment which had accumulated at the bottom of the flask during incubation. The shaken contents of the flask were then diluted with membrane-filtered "Pneumo-Broth" to provide final dilutions of 80, 60, 40 and 20 per cent of the bacterial culture. Ten ml. quantities of the undiluted bacterial culture and the above mentioned dilutions were placed in separate chemically-clean Evelyn absorption tubes which belonged to one standardized set. These tubes had been standardized with distilled water to an accuracy of plus or minus one galvanometer unit.

After the Evelyn Colorimeter had been turned on for ten minutes, so that "drift" would be minimized, a preliminary trial was made using a number 660 mμ filter and 10ml. undiluted broth. This filter provided a good range of deflection over the galvanometer scale and was used throughout this study. Before each reading was attempted, the tubes containing the bacterial samples were thoroughly twirled in order to suspend the bacteria evenly. Before and after the observations from each sample, the galvanometer was adjusted to 100 per cent transmission of light with a control tube containing broth alone. Measurements were made with the source of light set at normal operating intensity. Triplicate readings were made with each sample, and the amount of deflection of light transmission was recorded to the nearest

quarter of the galvanometer scale unit. The galvanometer readings were expressed in terms of optical density (O.D.=2-log"g" where galvanometer reading is "G").

Results:

These are reported in Table 14.

TABLE 14

TUBE NO.	Culture Per Cent Conc.	Optical Density	Expected Optical Density	Per Cent Cumulative Error Dev.
1	100	0.5086	-	-
2	80	0.4260	0.4069	4.6
3	60	0.3233	0.3151	5.9
4	40	0.2111	0.2035	1.1
5	20	0.1051	0.1017	3.3

Conclusions:

The figures in column "Per Cent Cumulative Error Deviation", were derived in the same manner as described in Part (A) of this experiment. They represent the following sources of error:

1 - The human factor error involved with dilution of samples, resuspension of organisms and the recording of the results.

2 - The error to be expected when dealing with an organism which grows in chains.

3 - The error inherent in the galvanometer itself.

From the data obtained it seems that several of these errors may cancel each other in a test such as this.

The greatest error obtained was that of 5.9 per cent from

the expected value, and a combined error of 7.0 per cent between any two values. The average error of deviation from the expected values for all the results measuring the bacterial turbidity, was 3.7 per cent.

Summary:

It thus appears that the relative number of organisms in various bacterial populations may be estimated rapidly and with a reasonable degree of accuracy by using the Evelyn Photoelectric Colorimeter.

2. A - Preparation of a Standard Inoculum.

A standard method was adopted for the preparation of all inocula used in this study. Its accuracy has been evaluated in an experiment which will be described later.

A single colony from the stock blood-agar plate was used to inoculate another such plate on which the inoculum was spread thoroughly to ensure the appearance of isolated colonies. The plate was incubated at 37°C. for 24 hours. At the end of this period a standard wire loop was used to transfer six colonies from this plate into a five ml. volume of "Pneumo-Broth", which in turn was incubated at 37°C. for six hours. Following incubation, the organisms were washed three times in triple-distilled water to ensure their freedom from substances present in the growth medium. This process was carried out as follows: The cells were centrifuged at 3,000 R.P.M. for 15 minutes, the supernatant fluid was removed and replaced by 10 ml. triple-distilled water along with three sterile glass beads. The contents of the tube were shaken in order to dislodge the sediment which appeared as a result of centrifugation. This procedure ensured a thorough washing, and was repeated three times. The final 10 ml. suspension was used as the source of the inoculum. Throughout this investigation 0.1 ml. of such a suspension was used to inoculate the test tubes.

In all cases the inoculum was checked microscopically for purity of the culture prior to inoculation of the test media, and at the time of their inoculation, a blood-agar plate was

spread with a drop of the suspension and incubated at 37°C. At the end of 24 hours, the plate was examined for the presence of contaminating colonies.

2. B - An Evaluation of the Constancy of the Standard Inoculum.

Introduction:

Since this study involved to a considerable extent the comparison of the amount of growth obtained in various media at different times, it was requisite that the inocula remain as constant as possible. This experiment was carried out, therefore, in order to determine the differences in the number of organisms obtained in several suspensions prepared simultaneously according to the standard method described previously.

Materials and Methods:

Ten individual inocula were prepared according to the previously described method, and each was then transferred to a chemically-clean absorption tube. Using a control tube consisting of 10 ml. distilled water, the amount of transmission was recorded to the nearest quarter of the galvanometer scale unit as a per cent, using the Evelyn Colorimeter in the manner described in Experiment 1.

Results:

These are presented in Table 15.

TABLE 15

Suspension No.	Per Cent Light Transmission
1 (control)	100.00
2	85.00
3	81.00
4	81.25
5	81.50
6	82.50
7	83.75
8	81.25
9	82.75
10	84.00
11	83.50

Mean per cent light transmission - 82.65 per cent
Mean per cent deviation $\pm$ 1.155 per cent

Conclusions:

It appears from this experiment that the standard method described for the preparation of the inocula, produces relatively constant results with respect to the number of organisms obtained. The results of the experiments to follow can therefore be regarded to some extent on a comparative basis.

3. - A Comparison Between Variously Supplemented Basal Media With Respect to Their Growth Supporting Abilities for the Test Organism..

Introduction:

Since one purpose of this study was to devise a medium as chemically-defined as would support a luxuriant growth of the experimental strain, the first step in this endeavour consisted of the development of a suitable basal medium.

The choice of a basal medium is governed generally by the following factors.

1 - The medium provided it has been adequately supplemented, should be able to support growth of the test organism within a reasonable interval following inoculation.

2 - The medium should allow the development of organisms whose morophology is typical for the strain.

3 - The medium should be one that can be easily prepared.

The following were used as sources of basal media and were tested on a comparative basis with respect to the above requirements.

Materials and Methods:

Medium I after Slade, Knox and Slamp (1951)

Medium II after Slade and Slamp (1955)

Medium III after Pappenheimer and Hottle (1940)

Medium IV after Difco-Amino Acid Assay Medium (1953)

The four above mentioned basal media were devised by including in each only those listed substances which were chemically defined, any complex organic material being omitted in each case.

The individual constituents which were common to all four of these media were used in the same concentration throughout, so as to maintain the quantitative aspect of the comparison as constant as possible. Such substances are noted in Table 16.

TABLE 16

Substance	Concentration in mg. per 100 ml.	Substance	Concentration in mg. per 100 ml.
DL-Alanine	40	L-Tyrosine	40
L-Arginine-HCl	40	DL-Valine	40
L-Cystine	20	Glutamine	10
Glycine	40	Adenine Sulphate	1.5
L-Histidine	20	Uracil	1.0
DL-Isoleucine	40	Glucose	1000
DL-Leucine	40	[x]Thiamine-HCl	.1
L-Lysine-HCl	40	[x]Ca Pantothenate	.1
DL-Methionine	20	[x]Riboflavin	.1
DL-Phenylalanine	30	[x]Nicotinic acid	.1
L-Proline	20	Mono-potassium Phosphate	54
DL-Serine	40	Magnesium Sulphate	5
DL-Threonine	40	Ferrous Sulphate	.5
DL-Tryptophane	40		

[x] These substances were added in the liquid state from stock solutions. All others were used in dry form.

In several cases the optical isomer of an amino acid used in this experiment was not that which the author originally suggested. However, it was thought impractical to satisfy this need in every case since conflicting requirements in this respect have been noted.

In addition to the ingredients present in all four media (Table 16), other substances were incorporated into them in various combinations, according to the requirements listed in the reference to each. Table 17, lists these additions.

TABLE 1

Medium I	Medium II	Medium III	Medium IV	Conc. in mg/100ml.
L-Glutamic			L-Glutamic	20
DL-Aspartic			DL-Aspartic	20
Asparagine		Asparagine	Asparagine	80
Guanine-HCl			Guanine-HCl	1
Xanthine			Xanthine	2
x Pyridoxine-HCl				.2
x Pyridoxal-HCl	Pyridoxal-HCl		Pyridoxal-HCl	.2
			x Pyridoxamine-HCl	.2
x Vitamin B_{12}				.0002
x Biotin		Biotin	Biotin	.01
Folic Acid			Folic Acid	2
Inositol				2
x p-Aminobenzoic acid			p-Aminobenzoic acid	.02
Choline Chloride				.5
Ascorbic Acid		Ascorbic Acid		10
Sodium Ethyl - Oxalacetate				50
Sodium Fumarate				50
			Sodium Acetate	4000
			Ammonium Chloride	600
			Dipotassium phosphate	1200
Disodium Phosphate	Disodium Phosphate	Disodium Phosphate		670
x Manganese Sulphate			Manganese Sulphate	.25
	x Manganese Chloride	Manganese Chloride		.25
x Zinc Sulphate	Zinc Sulphate	Zinc Sulphate		.10
		x Copper Sulphate		.01
Sodium Bicarbonate				50
		Calcium Chloride		2
		x Glutathione		.2
			Sodium Chloride	4

All substances were added in dry state with the exception of those noted by x. These were added in liquid form from stock solutions. A sufficient volume of triple distilled water was added to the four flasks so that the final volume in each was 100 ml. The flasks were warmed gently and shaken until all substances had dissolved; Medium Number II remained cloudy throughout the experimental period, probably due to the insolubility of the sodium fumarate and sodium exaloacetate.

The hydrogen ion concentration of each flask was adjusted to approximately pH 7.6 with N NaOH, using the Beckman pH Meter (See Appendix II, Part D).

Each of the basal media was then dispensed in six chemically-clean absorption tubes. The three tubes to serve as controls always received 10 ml. each, and the three to be inoculated 9.9 ml. each.

All tubes were steamed at 100°C. for one hour on three consecutive days, in between and following which they were refrigerated at 5°C. in the dark. This treatment is in general use for sterilizing broth which is to be used for supporting an abundant growth of the Group A streptococci. It appears therefore, that the foregoing procedure does little to harm or destroy any essential component of the medium; it was thus adopted for preliminary use in this study until such time that a growth-stimulating medium was established, whence the various methods of rendering it sterile were evaluated.

It is a generally accepted fact that the Lancefield Group A streptococci require the incorporation of a peptide or a peptide containing substance into a medium before they will multiply. Up to the present time all attempts to cultivate the Group A streptococci on a completely synthetic medium have been unsuccessful. In view of the recent report of Slade and Slamp (1955), ovalbumin seemed the most defined form of the growth-stimulating supplement requiredby these organisms. Crystalline bovine serum albumin Fraction V being available, it too was assayed for its growth promoting ability. Although these same authors reported that this substance produced only 20 per cent of the response obtained with ovalbumin, in the presence of their basal medium, it was hoped that in the presence of basal medium other than Number II, this substance might promote an increased response of the organisms.

Accordingly the six tubes of each basal medium were divided into three sets of two tubes each; one tube in each set to be inoculated, and one to be used as an uninoculated control. The tubes in each set were supplemented as follows:

Set 1 - Each tube received ovalbumin in a concentration of 0.15 per cent (W/V).

Set 2 - Each tube received Fraction V in a concentration of 0.15 per cent (W/V).

Set 3 - Neither tube received supplementation.

All tubes were steamed at 100°C. for 10 minutes. This

procedure was reported by Slade and Slamp (1955) to be essential for the activation of the ovalbumin and Fraction V.

The inoculum of strain #35 was prepared according to the standard method except that the final suspension consisted of a 5 ml. volume instead of 10 ml. The tube in each set containing 9.9 ml. of medium received .1 ml. of this suspension.

All tubes were incubated at 37°C. After 18, 24, and 40 hours incubation, the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter in the manner described previously.

Results:

The results are presented in Table 18.

Gram-stained preparations from all inoculated tubes were made at the termination of incubation. The organisms obtained from Media I and IV appeared to be swollen and the chains of organisms appeared notably shorter; whereas the organisms grown in Media II and III appeared to be normal both in diameter and chain length.

TABLE 18

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

Medium No.	Set No.	18 hours CONTROL	18 hours TEST	24 hours CONTROL	24 hours TEST	40 hours CONTROL	40 hours TEST
I	1	100	99.5	100	100	100	100
I	2	100	100	100	99.75	100	100
I	3	100	100	100	99.5	100	100
II	1	100	[x]89.5	100	[x]85.5	100	[x]81.0
II	2	100	98.25	100	98.0	100	98.0
II	3	100	99.0	100	98.5	100	98.25
III	1	100	97.5	100	97.0	100	97.0
III	2	[#]100	100+	100	100+	100	100+
III	3	100	98.75	100	98.5	100	97.5
IV	1	100	98.5	100	98.25	100	98.25
IV	2	100	99.0	100	98.5	100	98.5
IV	3	100	99.5	100	99.5	100	100

[x] This tube was contaminated.

[#] In the event of contamination of the control tube as was the case here, the per cent light transmission of the inoculated tube was above 100 on the galvanometer scale.

Legend to Table 18

Set No.	Supplement	Per Cent Conc. Of Supplement In Medium W/V
1	Ovalbumin	0.15
2	Fraction V	0.15
3	nil	-

Discussion:

From the results obtained it appears that Medium II and III are similar as they permit the growth of a small number of Gram-positive cocci in chains typical of the Type 12 streptococci. However, Medium III did contain substances which had previously been reported as essential for the growth of the Group A streptococci and which were absent from Medium II. For example, glutathione was reported by Pappenheimer and Hottle (1940), to be required by these organisms for growth. These authors do not fail to point out that any substance with reducing powers could serve the same function as glutathione. Medium II contained no such substance other than cystine, yet supported growth to the same extent as Medium III. In a medium which is incapable of supporting a sizeable growth, the need for a reducing agent is not as noticeable, since the cystine in the medium can satisfy the small demand for such an agent under these conditions. However in a medium capable of supporting a large number of organisms, a reducing agent other than cystine must be present if optimal growth is to be realized. A future experiment confirms this statement.

Neither ovalbumin nor Fraction V appears to offer any notable stimulus to growth in any of the media tested. The absence of growth in Medium II which was supplemented with ovalbumin cannot be readily explained, since as far as possible, the conditions employed in this case were identical to those which Slade and

Slamp (1955) reported as capable of promoting a sizeable yield of Group A streptococci. However these authors do mention a strain of Type 12 streptococcus which they could not grow under their experimental conditions.

Conclusions:

In view of the foregoing discussion, Medium III was adopted for use throughout this study since it appeared to fulfill the requirements expected of a basal medium.

Before a decision could be reached regarding the growth-supporting ability of either ovalbimin or Fraction V, each had to be further assayed in higher concentrations.

SECTION B

I. THE ABILITY OF VARIOUS ORGANIC COMPLEXES TO STIMULATE GROWTH OF THE TEST ORGANISM IN AN OTHERWISE DEFICIENT BASAL MEDIUM. PARTS 4-7 AND 10-11.

II. STERILIZATION OF MEDIA. PART 8.

III. THE EFFECT EXERTED BY TWO CONSTITUENTS OF THE BASAL MEDIUM. PART 9.

General Introduction:

A suitable basal medium having been established, itself incapable of supporting a luxurious growth of the test organism, the next step consisted of evaluating various forms of supplementation for their relative growth stimulating abilities in this basal medium.

Over the past two decades, various sources of the growth-stimulating supplement required by the Group A streptococci have been employed by several investigators. Smith (1937), reported results which suggested the extreme importance of peptone in supporting growth of these organisms. McIlwain in 1940 reported on the growth-stimulating activity provided by a hot extract of yeast. Woolley in 1941 made use of an aqueous liver extract called liver fraction "L" for supplementing a basal medium. In the latter two instances a preparation of casein hydrolyzate was also included in these media. The most recent report is that of Slade and Slamp (1955), who indicated that ovalbumin when suitably activated was capable of stimulating growth of several Group A streptococci in an otherwise inactive synthetic medium. It therefore seemed of utmost importance to determine which of these supplements was most active under the conditions of this experiment. Further, since nephrotoxin formation although not an inevitable accompaniment of streptococcal growth is dependent upon it, it is obvious that in a medium in which the organisms are capable of elaborating this substance, optimal growth will result in optimal

toxin formation. Thus the determination of the optimal concentration of any substance promoting growth, is of paramount importance, and hence the supplements have been assayed in various concentrations.

4. Ovalbumin and Fraction V

Introduction:

In the concentration used in the previous experiment, neither ovalbumin nor Fraction V proved itself to be more than slightly active as a growth-stimulant. However, ovalbumin is believed to serve two functions when used to supplement a medium, namely:

1 - It may play the role of a reducing agent by virtue of its content of sulfhydryl groups which are unmasked by heat treatment, ultra-violet irradiation, or by shaking.

2 - It may fulfill the requirements for growth of the Group A streptococci in an incomplete synthetic basal medium by virtue of its organic structure.

There remained, therefore, the possibility that the concentration of ovalbumin previously tested was inadequate to serve one or both of these functions, and it was considered reasonable to assume that an increase in the concentration of this substance in the basal medium might prove beneficial. Whether Fraction V exerts its stimulatory effect in a manner identical to ovalbumin has not been reported, however it was felt that it too should be reexamined for its growth-supporting ability in

higher concentrations than previously tested.

Materials and Methods:

One-hundred and twenty ml. of Medium III was prepared according to the procedure described in Section A (3), and was dispensed in 6 pairs of absorption tubes, one tube in each pair receiving 10 ml. and serving as a control, the other 9.9 ml. All tubes were sterilized in the usual manner. Ovalbumin or Fraction V was added to the pairs of tubes in the concentrations reported in the legend to Table 19.

All tubes were steamed at 100°C. for 10 minutes in order to activate the supplements, following which the tube in each pair containing 9.9 ml. of medium, was inoculated with 0.1 ml. of a standard inoculum. Incubation of all tubes at 37°C. followed. After 18, 24, and 40 hours incubation the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter.

Results:

The results are recorded in Table 19.

TABLE 19

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
PAIR NO.	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
1	100	99.0	100	99.25	100	98.75
2	100	98.5	100	97.75	100	98.25
3	100	x86.5	100	x80.75	100	x72.0
4	100	98.5	100	98.25	100	98.25
5	100	99.25	100	98.5	100	98.5
6	100	x88.75	100	78.25	100	69.0

Legend to Table 19

Pair No.	Supplement	Per cent Conc. Of Supplement In Medium (W/V)
1	Ovalbumin	0.2
2	"	0.4
3	"	0.8
4	Fraction V	0.2
5	"	0.4
6	"	0.8

x These tubes were contaminated.

5. Proteose Peptone and Yeast Extract

Introduction:

The foregoing experiment having indicated that ovalbumin and Fraction V, the simplest forms of supplementation so far reported for the Group A streptococci, are inactive under the conditions of this experiment, it was decided to assay more complex sources of the growth-stimulating factor.

Proteose peptone and yeast extract were the obvious choices since these substances have long been known as being efficient in promoting growth of these organisms in an otherwise insufficient basal medium.

Materials and Methods:

Medium III was prepared in a 280 ml. volume according to the procedure described previously. It was dispensed into 14 pairs of absorption tubes, one tube in each pair receiving 10 ml. of the medium and the other 9.9 ml. Proteose peptone or yeast extract was added to the pairs of tubes in the concentrations reported in the legend to Table 20. These concentrations were selected for testing in the hope that such a wide range would provide an indication as to the particular concentration of each substance which would be optimal for growth.

Since the addition of these substances to each tube caused a lowering of the pH of the media, N NaOH was used to readjust the pH of each to approximately 7.6, prior to sterilization in the previously described manner.

The tube in each pair containing 9.9 ml. of medium was inoculated with 0.1 ml. of an inoculum prepared according to the standard procedure described previously, excepting that the final suspension consisted of 5 ml. instead of 10 ml.

All tubes were incubated at 37°C. After 18, 24, and 40 hours incubation, the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter.

Results:

These are presented in Table 20.

LEGEND TO TABLE 20

Pair No.	Supplement	Per Cent Conc. Of Supplement In Medium (W/V)
1	Proteose peptone	0.2
2	"	0.4
3	"	0.8
4	"	1.0
5	"	2.0
6	"	4.0
7	"	8.0
8	Yeast extract	0.2
9	"	0.4
10	"	0.8
11	"	1.0
12	"	2.0
13	"	4.0
14	"	8.0

Table 20

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
PAIR NO.	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
1	100	92.0	100	86.5	100	85.25
2	100	89.0	100	85.5	100	83.0
3	100	85.0	100	81.5	100	80.25
4	100	80.25	100	73.5	100	72.25
5	100	76.0	100	69.0	100	66.5
6	100	59.5	100	56.5	100	52.0
7	100	73.5	100	72.0	100	71.0
8	100	97.5	100	96.0	100	95.0
9	100	73.5	100	71.0	100	70.5
10	100	69.0	100	67.5	100	64.25
x11	100	97.25	100	97.0	100	97.0
x12	100	96.5	100	95.5	100	94.0
x13	100	92.5	100	90.5	100	90.0
x14	100	91.5	100	89.5	100	87.5

[x] The figures in the last four pairs were obtained by making a 1:10 dilution of the well shaken cultures and their respective controls in distilled water at the various time intervals. This procedure was necessitated by the fact that these undiluted cultures gave readings in the Evelyn Colorimeter which did not fall on the galvonometer scale. It must be noted that these readings cannot be compared with those of the undiluted cultures, but are meaningful only within the group identically treated.

6. Liver Fraction "L" and Casein Hydrolyzate

Introduction:

In our search for the most effective growth-promoting supplement for the test organism, it was thought that both liver fraction "L" and casein hydrolyzate should be assayed under the conditions of this experiment, since various investigators have noted the activity of both of these substances in this regard.

Materials and Methods:

Medium III was prepared in a 160 ml. volume according to the procedure previously described. It was dispensed in 8 pairs of absorption tubes one tube in each pair receiving 10 ml. and the other 9.9 ml. The supplements were added to the various pairs of tubes in the concentrations reported in the legend to Table 21. The pH of each tube was then readjusted to approximately 7.6 on the Beckman pH Meter using N NaOH. All tubes were sterilized in the usual manner. The tube in each pair containing 9.9 ml. of medium was inoculated with 0.1 ml. of the standard inoculum following which all tubes were incubated at 37°C. After 18, 24 and 40 hours incubation, the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter.

Results:

The results are presented in Table 21.

TABLE 21

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
PAIR NO.	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
x1	100	98.25	100	96.25	100	95.0
x2	100	97.25	100	96.0	100	95.5
x3	100	94.0	100	92.0	100	91.25
x4	100	90.0	100	87.5	100	87.0
5	100	97.5	100	94.0	100	91.0
6	100	87.5	100	82.0	100	79.5
7	100	91.0	100	89.0	100	88.0
8	100	98.2	100	95.5	100	93.5

Legend to Table 21

Pair No.	Supplement	Per Cent Conc. of Supplement in Medium (W/V)
1	Liver fraction "L"	1.0
2	"	2.0
3	"	4.0
4	"	8.0
5	Casein hydrolyzate	1.0
6	"	2.0
7	"	4.0
8	"	8.0

[x] These results represent readings of a 1:10 dilution of the various cultures and their controls at the designated times. (See footnote to Table 20).

Conclusion:

Crystalline ovalbumin and Fraction V of bovine serum albumin when activated by heat under controlled conditions, failed to permit growth of this Group A streptococcus in the synthetic basal medium, in all concentrations used. It could very well be that these nitrogeneous supplements by virtue of their high degree of refinement were unable to provide these organisms with the stimulus essential for growth.

Each of the four organic complexes assayed in Parts 5 and 6 of this Section appeared capable of supporting a luxurious growth of the test organism especially when each was used in its optimal concentration.

From the results obtained, it can be postulated that both proteose peptone and casein hydrolyzate contain an "inhibitory" as well as a stimulatory fraction for the Group A streptococci. When the concentration of these supplements in the medium is low, the amount of growth obtained is small; with increasing concentrations a parallel increase in the amount of growth is observed up to the point where the concentration of the "inhibitory" fraction in the peptone or casein is high enough to overshadow the effect of a similarily high concentration of the stimulatory fraction. At this point the efficiency of the supplement as regards its growth-stimulating ability is reduced.

The amount of growth obtained with the various concentrations of yeast extract or liver fraction "L", strongly indicates

that little if any "inhibitory" substances are present in these complexes, since within the limits of this experiment, an increasing concentration of either of these substances was followed by an increased growth response of the organisms.

7. "B" Vitamins:

Introduction:

In view of the fact that the yeast extract (tested in (5.) of this section), proved efficient in supporting growth of the test organism, and since this form of supplementation is known to be a rich source of the "B" vitamins, it was considered of importance to determine whether it exerted its stimulatory effect by virtue of its high content of these vitamins or whether it was due to other growth-promoting substances which it may have contained.

Materials and Methods:

Medium III was prepared in a 20 ml. volume and dispensed in two absorption tubes, one receiving 9.9 ml. and the other 10 ml. To each was added a supplement consisting of various "B" vitamins in the following amounts:

	mg./ 10 ml.
Thiamine	3
Riboflavin	3
Pantothenic acid	3
p-Aminobenzoic acid	1
Pyridoxine	2
Pyridoxal	2
Choline Chloride	1
Inositol	1

The pH of each tube was readjusted to approximately 7.6 using N NaOH following which both were sterilized in the usual manner. The tube containing 9.9 ml. medium was inoculated with 0.1 ml. of the standard inoculum, and then both were incubated at 37°C. After 18, 24 and 40 hours incubation, the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter.

Results:

These are presented in Table 22.

TABLE 22

Age of culture	Control	Test
18 hours	100	97.5
24 hours	100	97.0
40 hours	100	97.0

Conclusions:

It appears that the supplement of "B" vitamins does not offer any stimulus to growth in the absence of other unidentified growth-stimulating molecules present in the yeast extract. It can thus be concluded that the latter substance exerts its activity almost entirely by virtue of its content of organic substances other than the "B" vitamins.

8. An Evaluation of the Various Methods Available for Sterilizing Media.

Introduction:

In order to be of any practical value, a medium must be sterilized prior to inoculation. This experiment was carried out to determine the most efficient method of sterilizing the basal medium when supplemented in various ways. Such a procedure should ensure the following:

1 - It should eliminate contaminating organisms.

2 - It should not in any way alter the components of the medium.

Todd and Hewitt (1932) pointed out that certain substances in a medium used for the growth of Group A streptococci could not withstand autoclaving. For example, substances such as cysteine and glutamine were found to be altered by this treatment. Pappenheimer and Hottle (1940) reported the same to be true for calcium pantothenate.

Three methods most commonly used for sterilizing media were thus compared with respect to the above requirements, as well as for their individual effect upon the basal medium which was supplemented in different ways. These methods were:

1 - Membrane Filtration.

2 - Autoclaving at 120°C. for 20 minutes.

3 - Steam at 100°C. for one hour on three consecutive days.

Materials and Methods:

Basal Medium III was prepared and divided into four aliquots of 60 ml. each. The aliquots were supplemented according

to the legends to Tables 23 to 26 and the pH of each adjusted to approximately 7.6 with N NaOH using the Beckman pH meter. Each medium was then further subdivided into three portions, each consisting of 20 ml.

Portion 1 of each medium was passed through a No. 5 filter membrane (see Appendix II, C.), and the filtrate collected in a sterile flask.

Portion 2 of each medium was autoclaved at 120°C. for 20 minutes.

Portion 3 of each medium was steamed at 100°C. for one hour on three consecutive days during and after which it was refrigerated at 5°C.

Following the various treatments each portion was dispensed in duplicate chemically-clean absorption tubes, one receiving 10 ml. and the other 9.9 ml. The tube in each pair containing 9.9 ml. of medium was inoculated with 0.1 ml. of the standard inoculum. All tubes were incubated at 37°C. After 18, 24, and 40 hours incubation, the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter. After 18 hours incubation a loopful from each tube was inoculated onto a blood-agar plate which was then spread and incubated at 37°C. for 24 hours. At the end of this period all plates were examined.

Results:

The amount of growth in the inoculated tubes is recorded in Table 23 to 26 respectively.

Examination of the blood-agar plates following incubation revealed pure cultures of beta hemolytic streptococci from those tubes which had been inoculated. All controls appeared sterile.

TABLE 23

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

Method of sterilization	18 hours		24 hours		40 hours	
	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
Membrane Filtration	100	98.5	100	98.0	100	97.0
Autoclave 120°C 20 min.	100	93.75	100	91.5	100	90.5
Steam 100°C. 1 hr. 3 days	100	90.25	100	89.5	100	88.0

Yeast extract was used in a concentration of 8.0 per cent (W/V), in the above tubes.

TABLE 24

x PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

Method of Sterilization.	18 hours		24 hours		40 hours	
	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
Membrane Filtration	100	98.0	100	97.0	100	96.5
Autoclave 120°C 20 min.	100	95.5	100	91.0	100	90.5
Steam 100°C. 1 hr. 3 days.	100	94.0	100	92.0	100	88.25

Liver fraction "L" was used in a concentration of 8.0 per cent (W/V), in the above tubes.

x These figures represent readings of a 1:10 dilution of the cultures and their respective controls in distilled water. (See footnote to Table 20).

TABLE 25

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
Method of Sterilization	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
Membrane Filtration	100	85.75	100	84.25	100	80.0
Autoclave 120°C 20 min.	100	46.50	100	43.25	100	42.25
Steam 100°C. 1 hr. 3 days.	100	48.0	100	47.5	100	46.0

Proteose peptone was used in a concentration of 4.0 per cent (W/V) in the above tubes.

TABLE 26

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
Method of Sterilization.	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
Membrane Filtration	100	90.5	100	87.0	100	85.0
Autoclave 120°C 20 min.	100	86.5	100	84.0	100	81.0
Steam 100°C. 1 hr. 3 days.	100	85.0	100	82.0	100	80.5

Casein hydrolyzate was used in a concentration of 4.0 per cent (W/V) in the above tubes.

Conclusions:

It can be concluded from this experiment that all three methods of sterilization were efficient in removing contaminating organisms. However, the membrane-filtered medium appeared to be inferior with respect to its growth-supporting ability in the case of all the supplemented media tested. This could very well have been due to the adsorption of one or more nutrients either partially or completely by the membrane, during the course of filtration. The autoclaved media appeared capable of supporting optimum growth of the test organism although the pH of the medium after autoclaving had dropped to between 7.24 - 7.36. As this is the most efficient and simplest method of imparting sterility to any medium, it was adopted for use in all experiments which followed, in preference to the steaming treatment.

There are three possible explanations for the apparent absence of harmful effects observed after autoclaving media containing heat-labile substances.

1 - It may be that during this treatment, the larger organic molecules, which in this case were present in the form of either peptone, yeast extract, or liver extract, serve as protection for these substances.

2 - It may be that the autoclaving process only effects the heat-labile substances to a point where they are still utilizable by the organisms.

3 - Another possibility is that the duration of this drastic treatment is not of sufficient length to destroy or alter the total amount of any heat-labile substance in the medium.

9. An Examination of the Role Played by Certain Reducing Agents Under Aerobic and Anaerobic Conditions

Introduction:

It is a well known fact that the Group A streptococci are essentially anaerobic organisms with respect to their enzyme constitution and consequently require the presence of a reducing agent when cultivated under aerobic conditions. It seemed reasonable to assume therefore, that if such substances were acting merely in this capacity, these organisms should be capable of growing equally well under anaerobic conditions in the absence of such agents. Two substances commonly employed for their reducing action are glutathione and ascorbic acid. The possibility that the latter may in some way act as a vitamin when incorporated into a medium and that glutathione may be utilized as a tripeptide for growth per se was examined.

Materials and Methods:

One hundred ml. of Medium III was prepared according to the procedure described in Section A, with the omission of glutathione and ascorbic acid. Proteose peptone was added to the basal medium in a 4 per cent concentration (W/V). The medium was dispensed in chemically-clean absorption tubes. The two control tubes received 10 ml. of the medium, whereas the remaining eight to be inoculated received the amounts noted in Table 27. All tubes were autoclaved at 120°C. for 20 minutes in accordance with (7.) of this Section.

One per cent (W/V) solutions of ascorbic acid and glutathione respectively were prepared in triple-distilled water. Each solution was filtered through a filter membrane, collected in a sterile flask, and added aseptically in 0.1 ml. amounts to the tubes according to Table 27.

Since both of the solutions were clear and colorless it was felt that a control tube which contained neither of these substances could be used without appreciably altering the results. The tubes containing less than 10 ml. of medium were inoculated with 0.1 ml. of the standard inoculum (See Table 27). Those tubes which were to be incubated anaerobically (See Table 27), were placed in a McIntosh and Fildes jar which was then provided with an atmosphere of hydrogen. (See Appendix II, E-1 for details). All tubes were incubated at 37°C., for 24 hours. At the end of this time the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter.

Results:

These are presented in Table 27.

TABLE 27

	AEROBIC					ANAEROBIC				
TUBE NO	1	2	3	4	5	6	7	8	9	10
BASAL MEDIUM (ml.)	10	9.9	9.8	9.8	9.7	10	9.9	9.8	9.8	9.7
ASCORBIC ACID (ml.)	0	0	.1	0	.1	0	0	.1	0	.1
GLUTHATHIONE (ml.)	0	0	0	.1	.1	0	0	0	.1	.1
INOCULUM (ml.)	0	.1	.1	.1	.1	0	.1	0	.1	.1
PER CENT LIGHT TRANSMISSION	100	82.0	71.0	70.0	70.0	100	71.5	81.5	72.5	77.0

Discussion:

Under anaerobic conditions optimum growth occurred in the absence of a reducing agent as well as in the presence of gluthathione. It is obvious therefore, that the only role which glutathione plays in a medium is that of a reducing agent. Ascorbic acid on the other hand appears to exert an inhibitory effect upon growth under anaerobic conditions. It seems that when glutathione is also present it neutralizes this effect to a certain extent. There is no obvious explanation for this.

Under aerobic conditions the growth of the test organism in the absence of a reducing agent is poor. The need for such an agent, however, appears to be a non-specific one as has been previously reported (McIlwain, 1940). The presence of the two reducing agents in the culture grown aerobically provides approximately the same effect as does either of these substances alone.

Thus it appears that the growth-stimulating activity of both glutathione and ascorbic acid in any medium, is dependent solely upon their ability to act as reducing agents.

10. The Nutritive Effect of the Dialyzable and Non-Dialyzable Fractions Respectively of Proteose Peptone, Liver Fraction "L" and Yeast Extract.

Introduction:

It is evident from (5. and 6.) of this Section that both proteose peptone and casein hydrolyzate contain a fraction which is inhibitory to the growth of the test organism. The presence of an "inhibitory" substance in peptones has been demonstrated by several workers. Smith (1937) showed clearly that neopeptone (Difco) was separable by dialysis into two portions with very dissimiliar properties, and that the "inhibitory" factor for Group A streptococci was contained in that portion diffusing most easily through cellophane. Avery (1952) reported the removal from peptone of "inhibitory" substances for B. subtilis, by dialysis.

The experiment to be discussed had many purposes. It was designed to confirm the work of Smith and Avery with respect to the molecular size of the "inhibitory" substance in the peptone being used in this study, and to determine which fraction of peptone provided the greatest stimulation for growth of the test organism. From the demonstrations of the above mentioned investigators that the dialyzable fraction of peptone contains the substance inhibitory for growth, it cannot be concluded that the fraction which does not pass through cellophane possesses greater growth-stimulating activity than either the undialyzed

peptone or the dialyzable portion. It would seem rather to be a balance between the stimulatory and inhibitory properties of the individual fractions which determines their growth supporting abilities.

Casein hydrolyzate was not assayed in this manner, since it consists of molecules of such a size as would pass through cellophane and thus the location of the inhibitory substance is evident.

Since one of the purposes of this study was to devise a medium as chemically - defined as possible, that is, one which would not only support a luxurious growth of the test organism, but which would contain a minimum amount of complex undefined substances, the possibility that dialysis of the complexes being used in this study might reveal fractions with vastly differing abilities to support growth, could not be overlooked. Such fractions could ultimately be compared with known growth-stimulating media. Hence even though neither the yeast extract nor the liver fraction "L" used in our study seemed to contain a growth inhibitory substance, it seemed reasonable to examine the two fractions of each which were separable by dialysis, for possible differences in their growth-stimulating ability.

Materials and Methods:

Medium III was dispensed into 18 pairs of absorption tubes, one tube in each pair receiving 10 ml. and one 9.9 ml. of the basal medium. The dialyzable and non-dialyzable fractions of the proteose peptone, yeast extract and liver fraction "L".

(See Appendix I, C. for details of their preparation) were added to the pairs of tubes in the concentrations reported in the legends to Tables 28 - 30.

The pH of each tube was readjusted to approximately 7.6 prior to sterilization by autoclaving at 120°C. for 20 minutes. The tube in each set containing 9.9 ml. of the medium was inoculated with 0.1 ml. of the standard inoculum. All tubes were incubated at 37°C. After 18, 24, and 40 hours incubation, the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter.

Results:

The results are reported in Tables 28 - 30 respectively.

TABLE 28

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
PAIR NO.	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
1	100	50.0	100	40.0	100	38.0
2	100	49.0	100	42.5	100	40.5
3	100	60.0	100	56.5	100	55.5
4	100	71.5	100	69.0	100	59.0
5	100	83.5	100	78.0	100	56.0
6	100	65.5	100	61.5	100	51.5

Legend to Table 28

Pair No.	Supplement	Per Cent Conc. Of Supplement In Medium (W/V)
1	Dialyzable [x] p.p.	2.0
2	"	4.0
3	"	8.0
4	Non-Dialyzable [x] p.p.	2.0
5	"	4.0
6	"	8.0

[x] proteose peptone.

TABLE 29

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
PAIR NO.	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
7	100	38.5	100	45.5	100	42.0
8	100	61.0	100	56.5	100	54.0
9	100	54.0	100	47.5	100	45.0
10	100	92.0	100	88.5	100	79.5
11	100	94.5	100	90.25	100	82.0
12	100	86.5	100	78.5	100	75.0

Legend to Table 29

Pair No.	Supplement	Per cent Conc. Of Supplement In Medium (W/V)
7	Dialyzable [x] y.e.	2.0
8	"	4.0
9	"	8.0
10	Non-Dialyzable [x] y.e.	2.0
11	"	4.0
12	"	8.0

[x] Yeast extract.

TABLE 30

PER CENT LIGHT TRANSMISSION

AGE OF CULTURE

	18 hours		24 hours		40 hours	
PAIR NO.	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
13	100	99.5	100	98.0	100	96.5
14	100	97.0	100	94.5	100	88.0
15	100	96.5	100	92.0	100	89.5
16	100	98.0	100	95.5	100	94.0
17	100	98.0	100	97.5	100	97.0
18	100	99.0	100	98.0	100	96.5

Legend to Table 30

Pair No.	Supplement	Per Cent Conc. Of Supplement In Medium (W/V)
13	Dialyzable [X] l.f. "L"	2.0
14	"	4.0
15	"	8.0
16	Non-Dialyzable [X] l.f. "L"	2.0
17	"	4.0
18	"	8.0

[X] Liver fraction "L".

Discussion:

The results obtained using the two fractions of proteose peptone indicate that only the dialyzable portion of this substance contains the growth - "inhibitory" factor in addition to its growth-stimulating ability. The non-dialyzable fraction also contains growth-stimulating substances, but a much greater concentration (W/V) of this fraction is required to support an optimal yield of the test organism than of the dialyzable fraction. Thus the indication is that the former fraction possesses a much smaller proportion per given weight of the growth-stimulating factor than does the latter.

It is also apparent from these results that the bulk of the growth in the tests of pairs 1 - 3 was obtained within the first 24 hours of incubation, whereas in the tests of pairs 4 - 6 most of the growth occurred after the 24th hour of incubation. It seems reasonable to assume therefore, that the growth-stimulating substances in the non-dialyzable fraction of proteose peptone were present as part of complex molecules which were inaccessable as such, for use as growth factors, and hence it was not until smaller fragments - the decomposition products of the larger molecules, had become available, that a sizeable yield of organisms was obtained.

In the case of the yeast extract the dialyzable fraction appeared to be somewhat superior in its growth-stimulating ability in comparison with that portion which was held back by the

cellophane during dialysis. Within the limits of the concentrations used in this study, an "inhibitory" fraction could not be detected. Also there was no evidence of an extended lag period in the medium containing the non-dialyzable fraction of yeast. This may have been due to one of two things. 1 - The organisms may have been able to utilize the larger molecules per se. 2 - Only a minimal amount of decomposition of the complex molecules may have been required to make the utilizable material available for the organisms; in other words, the groupings which the organisms required for growth may have been those on the outermost parts of the complex molecules and were therefore fragmented by the organisms during the first stages of incubation.

Our results with the two portions of liver fraction "L" were somewhat inconsistent with the report of Woolley (1941), He mentioned the presence of an "inhibitory" factor in the non-dialyzable portion of the liver. In our experiment the dialyzable fraction did appear to possess a greater ability to stimulate growth than did the non-dialyzable fraction, however, there was no evidence of an "inhibitory" factor in the latter.

11.-A Comparison Between the Amount of Growth Obtained in the Variously Supplemented Media With That in "Pneumo-Broth", and in Todd-Hewitt Broth, and the Effect of 10 Per Cent CO_2 on These Cultures During the Incubation Period.

Introduction:

The purpose of this experiment was to confirm and extend some of the observations made in the foregoing experiments, which at best were little more than preliminary in that although the optimal concentration of each supplement for growth was determined, the results obtained at different times could not be compared with one another due to the large number of variable factors involved in each testing. For example, the size of the inoculum although constant to a certain extent, was nevertheless subject to variations. The basal medium employed in each test although of a constant composition ranged in age from one to ten days. The pH of the autoclaved media was lower at the time of inoculation than that of the media that had not been subjected to this treatment. These variations and many others indicated that a true comparison between the amounts of growth realized in the presence of these different supplements can be obtained only when the tests are carried out simultaneously.

Further if any of the media developed in this study are to be of any practical value, they must support growth of the test organism to an equal or greater extent than that obtained in the media used routinely for the growth of the Group A streptococci.

Therefore "Pneumo-Broth" and Todd-Hewitt broth were included in this experiment on a comparative basis.

Since the presence of 10 per cent CO_2 in the atmosphere has long been reported as growth-enhancing for cultures of Group A streptococci, the effect of this factor under the conditions of this experiment was considered worthy of investigation.

Materials and Methods:

Medium III was prepared in a 360 ml. volume in the usual manner. It was dispensed into 18 pairs of absorption tubes, one tube in each pair receiving 10 ml. and the other 9.9 ml. of the medium. The pairs were grouped into 9 sets consisting of 2 pairs each. All tubes were supplemented according to the legend to Table 31. The concentrations of the various supplements which were used in this experiment were those found in the preceding experiments to be optimal for growth. The pH of each tube was adjusted to approximately 7.6 on the Beckman pH Meter using N NaOH. All tubes were then sterilized by autoclaving at 120°C. for 20 minutes, following which the pH of each tube was adjusted once more to 7.6. The tube in each pair containing 9.9 ml. of medium was inoculated with 0.1 ml. of the standard inoculum. One pair from each set was placed in a McIntosh-Fildes jar to which 10 per cent CO_2 was then added (See Appendix II, E (2)); all tubes were incubated at 37°C. After 18, 24 and 40 hours of incubation the amount of growth in the inoculated tubes was measured in the Evelyn Colorimeter.

Results:

These are presented in Table 31.

Legend to Table 31

Set No.	Supplement	Per cent conc. Supplement in Medium (W/V)
1	Proteose Peptone	4.0
2	Dialyzable x p.p.	2.0
3	Non-Dialyzable x p.p.	8.0
4	Yeast Extract	8.0
5	Dialyzable £ y.e.	2.0
6	Non-dialyzable £ y.e.	8.0
7	Liver Fraction "L"	8.0
8	Dialyzable # l.f. "L"	4.0
9	Casein Hydrolyzate	2.0
10	"Pneumo-Broth"	-
11	Todd-Hewitt Broth	-

x Proteose peptone

£ Yeast extract

Liver fraction "L"

TABLE 31

PER CENT LIGHT TRANSMISSION

	ATMOSPHERE						ATMOSPHERE 10 PERCENT CO_2					
	AGE OF CULTURE											
	18 hours		24 hours		40 hours		18 hours		24 hours		40 hours	
Set No.	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
1	100	56.0	100	52.25	100	50.0	100	26.0	100	21.5	100	16.75
2	100	52.0	100	49.25	100	33.75	100	47.75	100	35.25	100	30.5
3	100	67.75	100	61.5	100	53.0	100	55.0	100	45.25	100	41.25
x_4	100	92.25	100	90.5	100	89.5	100	91.25	100	90.0	100	90.0
5	100	52.0	100	45.5	100	44.0	100	38.0	100	27.0	100	22.5
6	100	82.75	100	75.5	100	72.0	100	77.25	100	64.0	100	61.0
x_7	100	89.25	100	89.0	100	88.0	100	91.5	100	91.0	100	91.0
x_8	100	97.0	100	91.5	100	89.0	100	97.0	100	90.75	100	88.25
9	100	86.5	100	81.0	100	78.75	100	85.5	100	82.5	100	76.5
10	100	33.0	100	31.5	100	31.0	100	39.0	100	30.5	100	27.5
11	100	83.1	100	82.5	100	80.0	100	67.5	100	67.0	100	66.0

^{x} These figures were obtained using a 1/10 dilution of the well shaken cultures and their respective controls. (See footnote of Table 20 for details).

Conclusions:

It is obvious from the above results that the presence of 10 per cent CO_2 in the atmosphere during incubation has a marked stimulatory effect on some of the media examined. This is particularly noticeable in the case of Sets 1, 3, and 11. In view of the fact that the effect of this factor is not consistently beneficial, or else not to the same extent in the variously supplemented media, there remains the possibility that the different response obtained with the various supplements tested is due to their different organic content. For example in the case of the yeast extract (complete) the high content of nucleic acids may have prevented the CO_2 from exerting any noticeable effect.

The overall optimal growth was obtained in the medium containing proteose peptone (complete), which had been incubated in the presence of 10 per cent CO_2; this yield of organisms was even larger than that obtained in either "Pneumo-Broth" or Todd-Hewitt broth.

Media supplemented with the dialyzable fraction of either proteose peptone or yeast extract also appeared capable of supporting growth to an equal or greater extent than either of the conventional above-mentioned media.

Thus media have been devised which are better defined chemically than those previously employed and which support high yields of the test organism.

Since as previously stated, the formation of "nephrotoxin" although not an inevitable accompaniment of active streptococcal growth is dependent upon it, the culture filtrates from these media which supported optimal growth were assayed biologically for the presence of this substance.

SECTION C.

THE BIOLOGICAL ASSAY OF

VARIOUS TYPE 12 CULTURE FILTRATES, FOR NEPHROTOXIN

12. The Effect in Rabbits of Intravenous Injections of Various Type 12 Culture Filtrates

Introduction:

The actual mechanism whereby the substance or substances elaborated by the streptococci produces acute glomerulonephritis is still unknown.

A current theory that has gained wide acceptance is that of Schwentker and Comploier (1939). They suggest that during the primary infection with the streptococcal organisms, a toxin elaborated by these cells causes slight damage to some of the renal tissue, which is insufficient to produce clinical signs other than albuminuria, which is seen regularly during the course of various streptococcal infections. The kidney cells however, contain a specific haptene group which combines with the toxin, a protein, the final product being a complete antigen. This antigen acting true to form, stimulates the production of kidney antibodies, which in turn attack the renal tissue, giving rise to the clinical and pathological pictures associated with acute nephritis. Their experimental work using kidney tissue mixed with bacterial toxins tended to support this theory.

A great variety of experimental attempts to produce acute glomerulonephritis comparable to the clinical and pathological pictures in man, have been reported. However, it is only recently that encouraging results have been obtained by Reed and Matheson (1954). Working with a nephritogenic strain of Type 12 streptococcus, they were able to produce

hematuria, albuminuria, and hypertension in rabbits, by intravenous injection of a cell-free filtrate. Hypertension developed in the animals approximately 15 days following the first injection of the filtrate. Kidney lesions were primarily tubular, of the type associated with lower nephron nephrosis. Local infection with this Type 12 strain produced a similar picture. However with other serological types of Group A streptococci and with unrelated bacteria, clinical or pathological changes failed to appear.

The experiment to be reported was undertaken in order to determine whether the nephrotoxic substance had been elaborated and was present in the filtrates of the Type 12 cultures grown in the various media described in the foregoing Section.

Materials and Methods:

Animals:

Twelve rabbits each weighing between 4 and 5 pounds were used in this experiment. They were maintained on a diet of Purine rabbit chow and were given water freely.

Culture Filtrates:

The Culture filtrates were prepared by inoculating triplicate 35 ml. volumes of medium III each supplemented according to Table 32, with 0.35 ml. of the standard inoculum.

TABLE 32

Filtrate No.	Supplement	Per Cent Conc. of Supplement in Med.W/V
I	Dialyzable [x] p.p.	2.0
II	Dialyzable [£] y.e.	2.0
III	Proteose Peptone	4.0

[x] proteose peptone

£ yeast extract

Following 24 hours incubation at 37°C. each culture was placed in centrifuge tubes and centrifuged at 3,000 R.P.M. for 15 minutes, The supernatant fluid from each tube was passed through a filter membrane (See Appendix II C.). The resulting filtrates were tested for sterility by the inoculation of 0.5 ml. of each onto a blood-agar plate which was incubated for 24 hours and then checked for the presence of beta-hemolytic streptococci or for that matter any contaminating colonies.

The culture filtrates were stored in the refrigerator at 5°C., until 30 minutes prior to inoculation.

Inoculation Scheme and Schedule:

The twelve animals were divided into four groups of three.

GROUP I received intraveneous injections of sterile Filtrate I described in Table 33.

GROUP II received intraveneous injections of sterile Filtrate II described in Table 33.

GROUP III received intraveneous injections of sterile Filtrate III described in Table 33.

GROUP IV received no treatment whatsoever. They were used as uninoculated controls.

TABLE 33

Schedule of Injection of Filtrates

Day	Vol. injected (ml.)
1	1
4	1
7	1
10	2
13	2
16	3

The following procedure was carried out throught the duration of this experiment.

Collection of Urines:

Rabbits were placed in metabolism cages every two to five days. On each occasion they remained there until a minimum of 10 ml. of urine had been collected in the glass bottle below the cage. This could usually be accomplished overnight. It was assumed that such a volume would provide a sufficient concentration of any pathological constituents present.

Urinalysis:

The urines were shaken and the 10 ml. sample from each animal was placed in a conical graduated centrifuge tube and centrifuged for 30 minutes at 3,000 R.P.M. The supernatant fluid and sediment were separated for examination.

1 - Sediment: The sediment was examined microscopically, using the high power objective, for the presence of pathological constituents, namely red blood cells and casts.

2 - Supernatant Fluid: The supernatant fluid was examined for the presence of albumin. An accurate method for determing albumin in small quantities was reported by Kingsbury et al. (1926). The method consisted of the following steps:

1 - Preliminary Test

(a) To 2 ml. of the clear urine, 5 drops of 20 per cent sulphosalicylic acid was added as a preliminary test. If a white cloudiness appeared due to precipitation of albumin, a quantitative test was performed.

2 - Quantitative Test

(a) To 2.5 ml. of clear urine placed in a special comparison tube, 3 per cent sulphosalicylic acid was added to bring the final volume to 10 ml. The tube was inverted to mix the contents and then allowed to stand for 10 minutes.

(b) The turbidity in the tube was then compared visually with a set of seven standards which represented concentrations of albumin from 0.01 mg. per cent to 0.1 mg. per cent. A black line on a white background facilitated this comparison.

Measurement of The Arterial Blood Pressure of the Rabbits.

Following the original suggestion of Reed and Kropp (1944), a rabbit-holder was used which had been made by shaping plaster of paris bandages about a rabbit held in a crouched

position. The holder was then cut into an upper and lower portion and further strengthened by additional layers of plaster of paris bandages. A rabbit placed in this container could be maintained in a firm position, which was at the same time comfortable for him. The animals quickly became accustomed to this apparatus and offered no opposition to it. Thus the arterial blood pressure was taken under conditions which provided a minimum of stress to the animal.

The method used in this investigation to measure the peripheral arterial blood pressure of a rabbit has been described in detail by Reed and Matheson (1954). It is a modification of the ear capsule method originally suggested by Reed and Kropp (1944). Figure 1 illustrates the apparatus used for this purpose.

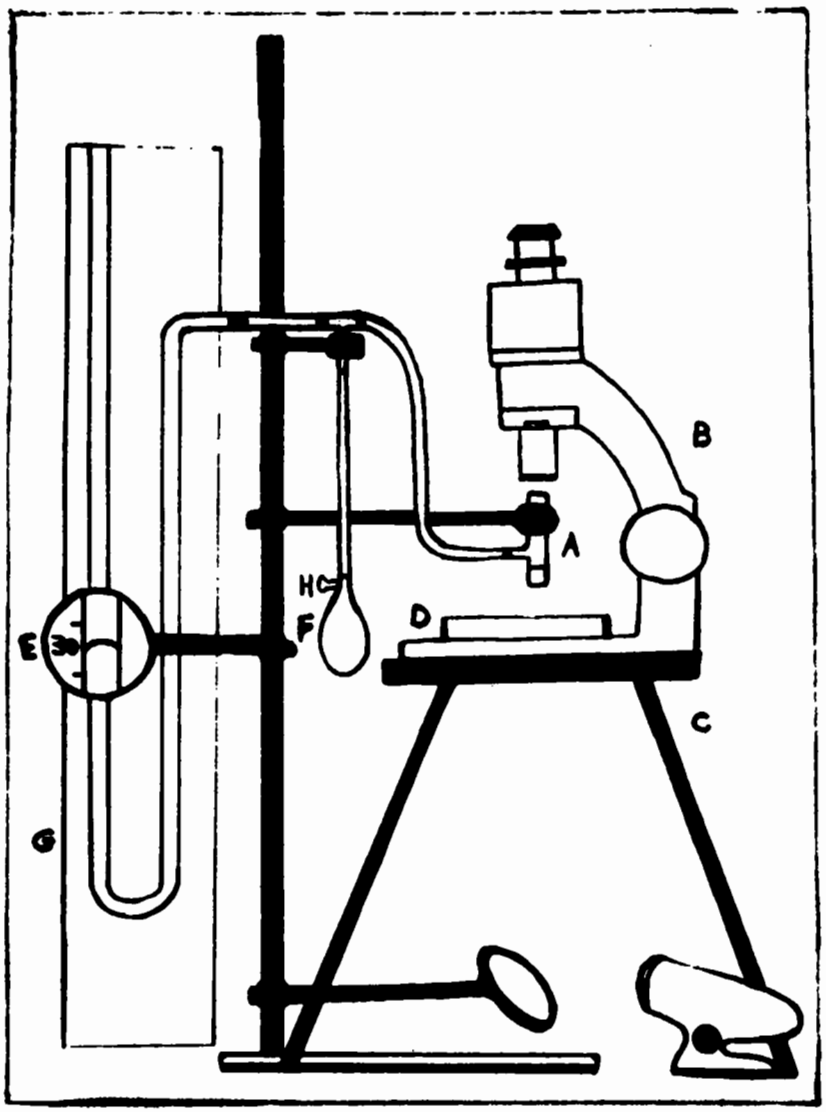

FIGURE 1.

This apparatus in figure 1 consisted of a brass tube (A) sealed at the upper end by a transparent glass disc, and at the lower one by an elastic condom rubber membrane, fastened in place with several layers of elastic bands which were then covered by a liquid rubber moulding compound. The brass tube was also fitted with a side-arm which connected to a manometer and a rubber bulb, by means of rubber tubing. Tube A was fastened with the glass disc upper most to a stereoscopic

microscope (B), which was connected to an adjustable screw clamp on a ring stand (C). On the platform of B rested a plexiglass plate (D). The lower portion of the ring stand was fitted with an adjustable screw clamp which supported an Abbé illuminator. This was used in conjunction with a light source to provide optimal light intensity at the site of the pressure reading. A magnifying glass (E) was placed in front of the manometer graduated in millimeters, to facilitate reading of the pressure.

The rabbit was placed in the plaster of paris mold described previously. The ear opposite to that receiving the injection was placed on D. Tube A was lowered until it barely touched the ear. The site at which the measurements were recorded was where the central artery branched.

The exertion of pressure on the bulb (F) when the needle-valve (H) was tightened caused the rubber membrane to become distended which resulted in the occlusion of the artery beneath it. As the pressure in A decreased slowly (1mm. per minute), the membrane began returning to its original state, thus releasing the pressure on the artery and allowing passage of blood through it. At this moment the pressure reading on the mercury manometer was recorded.

Ten consecutive readings were taken for each animal and the average was recorded.

Results:

Peripheral Arterial Blood Pressure

The peripheral arterial blood pressure of each animal was recorded over a 26 day period.

When the [x]control values for each animal were compared to those recorded during the latter part of treatment and following it, it became apparent that no significant rise in the blood pressure of any treated animal had occurred. Even when a slight rise was observed in this group, it could be correlated in most instances with a concurrent rise in pressure of the untreated animals.

Urinalysis

The majority of urines examined at various times during the 26 day period were found to be free from pathological constituents and albumin; however the following exceptions were observed.

Day 7 - One animal being treated with Filtrate III showed albumin in a concentration of 0.02 per cent.

Day 16 - One animal being treated with Filtrate II showed albumin in a concentration of 0.01 per cent.

Day 20 - One animal which was receiving no treatment showed albumin in a concentration of 0.01 per cent.

[x] The control period consisted of days one to five. According to Reed (1956), the blood pressure at this time is unaffected by the initial injections.

Conclusions:

The results of this experiment clearly indicate that none of the culture filtrates tested contained the nephritogenic substance, or if any of them did, it was in a concentration too low to be detected.

Although the supplemented media by virtue of their organic content were capable of supporting optimal growth of the test organism, they were in all probability deficient with respect to the molecules required for the biosynthesis of "nephrotoxin". It may very well be that a combination of the various supplements employed in this study will prove efficient in both respects.

SUMMARY

1. The Evelyn Colorimeter appeared to be an efficient instrument for measuring the amount of growth in various Type 12 populations.

2. A basal medium was devised which contained the various substances previously reported as being essential for growth of the Group A streptococci, as well as a variety of substances which are generally regarded as being growth-stimulating for microorganisms. This synthetic medium was itself incapable of inducing growth of the test organism.

3. The basal medium when supplemented with either ovalbumin or Fraction V of bovine serum albumin failed to induce a satisfactory growth response, regardless of the concentration used.

4. Proteose peptone, yeast extract, liver fraction "L", and casein hydrolyzate when used to supplement the basal medium were all found to be efficient in stimulating growth. The optimal concentration of each of these substances was determined.

5. The dialyzable and non-dialyzable fractions of each of the above mentioned substances (with the exception of casein hydrolyzate) were found in some cases to possess different growth-supporting abilities, while in other cases there were no striking differences observed between these two fractions.

6. The undialyzed complexes listed above, and the two fractions of each separable by dialysis, were tested on a comparative basis for their relative growth-supporting abilities. In some cases a fraction of a complex displayed a growth-stimulating ability

equal to or supperior to the undialyzed material. In view of the fact that both fractions of each complex as well as the original undialyzed material were growth-stimulating, there is every indication that this effect is due not to a single peptide or polypeptide but rather to a group of such molecules with a particular configuration.

7. The effect of the presence of 10 per cent CO_2 in the atmosphere during the incubation period was found to have a striking stimulatory effect on some cultures and a lesser effect on others depending upon the form of supplementation present in the medium.

8. The basal medium supplemented in three different ways was used for the preparation of culture filtrates to be assayed biologically for the presence of the nephritogenic substance. None of these filtrates was found to contain a detectable amount of this material.

9. All of the variously supplemented media were able to withstand autoclaving at 120°C. for 20 minutes.

10. Both gluthathione and ascorbic acid were found to be active in the medium exclusively by virtue of the ability of each to act as a reducing agent.

BIBLIOGRAPHY

Avery, R.J. M.Sc. Thesis, McGill University, Montreal. (1949).

Bainbridge, F.A. 1911 The action of certain bacteria on proteins. J. Hyg., 11, 341-355.

Bass, A., and Berkman, S., and Saunders, F. 1941 An additional growth factor needed by some hemolytic streptococci. J. Infect. Dis., 68, 220-225.

Bernheimer, A.W., and Pappenheimer, A.M. Jr. 1942 Factors necessary for massive growth of group A hemolytic streptococcus. J. Bact., 43, 481-494.

Bernheimer, A.W., and Rodbart, M. 1948 The effect of nucleic acids and of carbohydrates on the formation of streptolysin. J. Exp. Med., 88, 149-168.

Bernheimer, A.W. 1949 Formation of a bacterial toxin (streptolysin S) by resting cells. J. Exp. Med., 90, 373-392.

Difco Laboratories 1953 Difco mannual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures. 9 th ed. 230-236.

Fildes, P. 1934 Some medical and other aspects of bacterial chemistry. Proc. Roy. Soc. Med., 28, 79-90.

Freedman, L., and Funk, C. 1922(a) Nutritional factors in the growth of yeasts and bacteria. I. Vitamines. J. Metab. Res., 1, 457-468.

Freedman, L., and Funk, C. 1922(b) Nutritional factors in the growth of yeasts and bacteria. II. Protein Hydrolyzates. J. Metab. Res., 1, 469-480.

Friedman, T.E. 1939 The carbohydrate metabolism of streptococci. J. Biol. Chem., 130, 757-761.

Fruton, J.S. 1950 The role of proteolytic enzymes in the biosynthesis of peptide bonds. Yale J. Biol. Med., 22, 263-271.

Funk, C., and Dubin, H.E. 1920 A test for antiberri-beri vitamine and its practical application. J. Biol. Chem., 44, 487-498.

Funk, C. and Dubin, H.E. 1921 Vitamine requirements of certain yeasts and bacteria. J. Biol. Chem., 48, 437-443.

Grossiwcz, N. 1942 A new growth factor for hemolytic streptococci. Proc. Soc. Exp. Biol. Med., 49, 8-11.

Hanes, C.S., and Hird, F.J.R., and Isherwood, F.A. 1950 Synthesis of peptides in enzymic reactions involving glutathione. Nature, 166, 288-292.

Hosoya, S., and Kuroya, M. 1923 Water soluble vitamine and bacterial growth, with special reference to the chemical and physical properties of vitamin essential for the growth of hemolytic streptococci. Gov. Inst. Infect. Dis. Tokyo Imp. Univ., 2, 265-304.

Hottle, G.A., and Lampen, J.O., and Pappenheimer, A.M. Jr. 1941 Biotin as a growth factor for C 203 S strain of hemolytic streptococcus, Group A. J. Biol. Chem., 137, 457-458.

Hutchings, B.L., and Woolley, D.W. 1939 Growth of some hemolytic streptococci on a chemically defined medium. Science, 90, 41-42.

Hutner, S.H. 1938 Experiments on the nutrition of streptococci. J. Bact., 35, 429-440.

Ivanovics, G., and Euler, H. 1950 Some observations on the cultivation of streptococcus haemolyticus in chemically defined media. Biol. Abstr., 24, 1899.

Kingsbury, F.B., and Clark, C.P., and Williams, G. 1926 The rapid determination of albumin in the urine. J. Lab. & Clin. Med., 11, 981-989.

Mueller, J.H. 1920 Observations on bacterial metabolism. Proc. Soc. Exp. Biol. Med., 18, 14-17.

Mueller, J.F. 1921 Growth-determining substances in bacteriological culture media. Proc. Soc. Exp. Biol. Med., 18, 225-228.

Mueller, J.H. 1922(a) Studies in cultural requirements of bacteria. I.J. Bact., 7, 307-324.

Mueller, J.H. 1922(b) Studies on cultural requirements of bacteria. II. J. Bact., 7, 325-338.

Mueller, J.H. and Klise, K.S. 1932 Mass cultures of streptococcus Hemolyticus in broth. Proc. Soc. Exp. Biol. Med., 29, 454-455.

Murray, E.G.D., and Ayrton, R. 1924 Observations on the growth of Meningococci in vitro in relation to virulence. J. Hyg., 23, 23-63.

McIlwain, H., and Fildes, P., and Gladstone, G.P., and Knight, B.C.J.G. 1939(a) XXVII. Glutamine and the growth of streptococcus haemolyticus. Biochem. J., 33, 223-229.

McIlwain, H. 1939(b) Pantothenic acid and the growth of streptococcus haemolyticus. Brit. J. Exp. Path., 20, 330-333.

McIlwain, H. 1940 The nutrition of streptococcus haemolyticus. Growth in a chemically defined mixture; need for vitamin B_6. Brit. J. Exp. Path., 21, 25-38.

McIlwain, H. 1946 The metabolism and functioning of vitamin-like compounds. 3. Products of the decomposition of glutamine during streptococcal glycolysis. Biochem. J., 40, 460-464.

Pappenheimer, A.M. Jr., and Hottle, G.A. 1940 Effect of certain purines and CO_2 on growth of strain of group A hemolytic streptococcus. Proc. Soc. Exp. Biol. Med., 44, 645-649.

Rane, L., and Subbarow, Y. 1938 Studies on the nutritional requirements of hemolytic streptococci. I. effect of various substances isolated from liver extract on hemolytic streptococci. Proc. Soc. Exp. Biol. Med., 38, 837-839.

Reed, G.B., and Kropp, B. 1944 A simple apparatus for determining peripheral arterial pressure in the rabbit's ear. J. Lab. & Clin. Med., 29, 214-217.

Reed, R.W. 1953 An epidemic of acute nephritis. Canad. Med. Assoc. J., 68, 448-455.

Reed, R.W.,and Matheson, B.H. 1954 Experimental nephritis due to type specific streptococci. I. The effect of a single exposure to type 12 streptococci. J. Infect. Dis., 95, 191-201.

Reed, R.W. 1956 Personal Communication.

Robinson, H.C., and Rettger, L.F. 1918 The growth of bacteria in protein-free enzyme-and acid-digestion products. J. Bact., 3, 209-229.

Schwentker, F.F., and Comploier, F.C. 1939 The production of kidney antibodies by injection of homologous kidney plus bacterial toxins. J. Exp. Med., 70, 223-230.

Slade, H.D., and Knox, G.A. 1950 Nutrition and the role of reducing agents in the formation of streptolysin "O" by group A hemolytic streptococci. J. Bact., 60, 301-310.

Slade, H.D. 1951 Amino acid composition of a peptide mixture stimulatory for the growth of a group A hemolytic streptococcus. Bact. Proc., 76.

Slade, H.D., and Knox, G.A., and Slamp, W.C. 1951 The amino acid nutrition of group A hemolytic streptococci, with reference to the effect of glutathione on the cystine requirement. J. Bact., 62, 669-675.

Slade, H.D. 1954 The metabolism of amino acids by streptococci. Streptococcal infections; McCarty, M. Columbia University Press, New York.

Slade, H.D. and Slamp, W.C. 1955 The requirement of ovalbumin for the growth of group A hemolytic streptococcus in a synthetic medium. J. Exp. Med., 102, 291-305.

Smith, F. 1937 Peptone and production of haemolysin by streptococci. J. Bact. 34, 585-601.

Snell, E.E. 1951 Bacterial nutrition; chemical factors in bacterial physiology. Werkman, C.H., and Wilson, P.W. Academic Press, New York. 214.

Sprince, H., and Woolley, D.W. 1944 Relationship of a new growth factor required by certain hemolytic streptococci to growth phenomena in other bacteria. J. Exp. Med., 80, 213-217.

Stephenson, M. 1948 Bacterial metabolism. Longmans Green and Co. Toronto. 212.

Subbarow, Y., and Rane, L. 1939 Pantothenic acid as a growth factor for the Dochez N Y 5 strain of hemolytic streptococcus. J. Am. Chem. Soc., 61, 1616.

Todd, E.W., and Hewitt, L.F. 1932 A new culture medium for the production of antigenic streptococcal haemolysin. J. Path. and Bact., 35, 973-974.

Todd, E.W. 1938 Lethal toxins of haemolytic streptococci and their antibodies. Brit. J. Exp. Path., 19, 367-378.

van Niel, C.B. 1944 Recent Advances in our knowledge of the physiology of micro-organisms. Bact. Rev., 8, 225-234.

Williams, R.H. (See Subbarow and Rane 1939).

Wilson, A.T. 1945(a) Method for testing in vitro resistance of group A hemolytic streptococci to sulfonamides. Proc. Soc. Exp. Biol. Med., 58, 130-133.

Wilson, A.T. 1945(b) Nucleic acid derivatives as growth factors for certain group A hemolytic streptococci. Proc. Soc. Exp. Biol. Med., 58, 249-254.

Woolley, D.W., and Waismann, H.A. 1939 Nature and partial synthesis of the chick antidermatitis factor. J. Am. Chem. Soc., 61, 977-978.

Woolley, D.W., and Hutchings, B.L. 1939 Some growth factors for hemolytic streptococci. J. Bact., 38, 285-292.

Woolley, D.W. 1941 A new growth factor required by certain hemolytic streptococci. J. Exp. Med., 73, 487-492.

Woolley, D.W. 1946(a) Some correlations of growth-promoting powers of proteins with their strepogenin content. J. Biol. Chem., 162, 383-388.

Woolley, D.W. 1946(b) Strepogenin activity of serylglycyl-glutamic acid. J. Biol. Chem., 166, 783-784.

Woolley, D.W. 1948 Strepogenin activity of derivatives of glutamic acid. J. Biol. Chem., 172, 77-81.

Woolley, D.W., and Merrifield, R.B., and Ressler, C., and duVigneaud, V. Strepogenin activity of synthetic peptides related to oxytocin. Proc. Soc. Exp. Biol. Med., 89, 669-673.

APPENDICES

Appendix I.

The Preparation of Media and Various Materials to be Utilized in This Study.

A - 1. Pneumococcal Broth (McGill):

Finely minced beef heart freed of fat, is added to distilled water. Five hundred grams are required for each litre of distilled water. The mixture is heated to 75°C., to 80°C. for one hour and a half. The mixture is then filtered through filter paper pulp under slight suction.

Add proteose peptone (Difco) to a concentration of two per cent. Adjust the reaction to pH 7.2 using 10 N sodium hydroxide and phenol red as an indicator.

Heat at 120°C. for ten minutes. Cool the mixture and then filter through No. 1 Whatman filter paper. Adjust the pH to 8.0 with sodium hydroxide. Heat to 120°C. for ten to fifteen minutes. The mixture is then filtered again through filter paper pulp under slight suction.

This stock medium is bottled and autoclaved at 120°C. for 20 minutes.

Before the medium is distributed into tubes the following sterile ingredients are added under aseptic conditions; glucose at 0.1 per cent concentration, K_2HPO_4 at 0.2 per cent concentration, and fresh horse serum at 5.0 per cent concentration.

2. Blood-Agar Plates:

Finely minced beef heart, freed of fat, is added to distilled water. Five hundred grams of minced heart are required

for each litre of distilled water. The mixture is heated at 75°C. to 80°C. for one and a half hours. The fluid extract is filtered through filter paper pulp under slight suction.

Agar at a concentration of 1.5 per cent is dissolved, by autoclaving if necessary, in one-fifth the volume of the meat infusion, to which is added proteose peptone and the stock solution of salts (made up of sodium chloride 25 per cent, potassium chloride 2 per cent, and calcium chloride 1 per cent) to make one per cent in the final volume. Add the remaining four-fifths of the infusion, still at a temperature of 75°C. to 80°C., stir well and set aside in the cold overnight to gel. (Murray and Ayrton 1924).

The following day the gel is melted at 100°C. and the reaction adjusted to pH 8.5 with 10 N sodium hydroxide. Heat the bulk to 120°C. in the autoclave for 20 minutes to precipitate the phosphates; a visible precipitate should be produced. Filter through a paper pulp filter under slight suction. Adjust the reaction to pH 7.2 with 1N hydrochloric acid.

Check the pH of the bulk after adding a calculated amount of reagent; this is very important as the physical character of the agar alters the conditions of the neutralization, so that more than the calculated amount is usually necessary. Bottle and sterilize at 120°C. in the autoclave for 20 minutes. Cool this peptone agar medium to about 50°C., add defibrinated bovine or citrated human blood at a concentration of five per cent. Pour plates; incubate overnight.

3. Todd Hewitt Broth:

Finely minced beef heart freed of fat, is added to distilled water. One pound is required for each 1050 ml. of distilled water. The mixture is stirred and the fat particles skimed off using a sieve. The mixture is placed in an icebox overnight.

The following day the mixture is heated to 85°C. for one-half an hour and then filtered through course filter paper under slight suction.

For each litre, 20 gm. of neopeptone are added. The pH is adjusted to 7.0 with N NaOH, and the following ingredients added.

1. NaCl 2.0 gm.
2. $NaHCO_3$ 2.0 gm.
3. Na_2HPO_4 1.0 gm.
4. Glucose 2.0 gm.

Boil for 15 minutes and filter. Tube and sterilize in an autoclave by steaming for one hour on three consecutive days.

Organic Complexes:

The following Substances Were Used in This Study.

B - 1. Crystalline Ovalbumin - Nutritional Biochemicals Corp.

2. Bovine Albumin Serum Fraction V - Difco. Control #102969

3. Proteose Peptone-Difco. Control # 427606

4. Yeast Extract - Difco. Control # 424334

5. Liver Fraction "L" - Nutritional Biochemicals Corp. A dry concentrate of the alcohol insoluble fraction of liver.

6. Casein Hydrolyzate - Nutritional Biochemical Corp. A hydrochloric acid hydrolyzate, salt-free, and vitamin - free.

C - Dialysis:

The dialyzable and non-dialyzable fractions of the latter 4 aforementioned substances were prepared in the following way.

A 15 gram sample of each was brought into solution with 250 ml. of triple-distilled water.

Aliquots consisting of 125 ml. each were placed in a cellophane bag which was tied securely at both ends with string to prevent leakage of the fluid at the joints. Each bag was dialyzed twice for a 72 hour period against 2.5 litres of triple-distilled water. It was assumed that dialysis for this duration of time would ensure as complete a separation of the two dissimilar fractions as was possible.

1. Residue:

The residue in each bag was lyophilized from the deep frozen state, and stored at room temperature.

2. Dialyzate:

The five litres of dialyzate obtained from each substance was concentrated in the Laboratory Flash Evaporator, described in detail in Appendix II. The final volume of each complex prior to lyophilization from the frozen state was approximately 250ml.

D - Treatment of Water:

On most occasions during the course of this study the water which was used was that which has been referred to as being triple-distilled. It was obtained in this state by passage through a Research Model Illco-Way De-Ionizer, supplied by the Illinois Water Treatment Co. of Rockford Illinois.

This apparatus consists of a cartridge unit containing a specially dyed resin, and has an effect on water comparable to that produced by triple-distillation. It removes all ionizable solids, including Silica and CO_2. Its capacity is 450 grains as $CaCO_3$. Water which has been passed through this cartridge has a pH of 8.0 but readily absorbs CO_2 and the pH drops to approximately 7.5.

E - Glassware:

All pipettes, flasks, and tubes used in this study were soaked in nitrous acid for 48 hours, rinsed twice in distilled water and twice more in triple-distilled water, drained and dryed in a hot air oven.

Appendix II

The Description and Operation of Various Instruements & Materials Used in This Study

A - Freeze Dryer:

Lyophilization of all substances noted was carried out in a Centrifugal Freeze Dryer, Model 3 P.S. supplied by W. Edwards & Co. (London) Ltd.

B - Evelyn Photoelectric Colorimeter:

The measurement of all bacterial growths obtained in this study was carried out turbidimetrically using the Evelyn Photoelectric Colorimeter. This instrument was obtained from the Rubicon Co. Philadelphia Pa., and was accompanied by a Bulletin # 460 and a brochure, "Notes On Operation", which provided a complete description of the apparatus.

The tubes which were used in conjunction with this instrument were specially selected absorption tubes measuring 7" X 7/8". Prior to use all tubes were washed in liquid soap with a cloth covered brush (to avoid scratching) rinsed, and then allowed to soak in nitrous acid for 48 hours. After this treatment they were rinsed twice in distilled and twice in triple-distilled water, drained and dried in a hot air oven.

Care was taken to avoid soiling or scratching the lower portion of these tubes by surrounding this area with parchment paper jackets.

Guaze covered cotton plugs were used for all tubes.

C - Filtration Apparatus:

Filtering procedures carried out in this study involved the use of filter membranes which have an extremely uniform microstructure and a very smooth surface. They are produced from pure cellulose or cellulose derivatives. Those filter membranes listed as "Coli" 5 in the catalogue of Schleicher & Schuell Co. Keene, N.H. being of medium porosity (0.75 - 0.5 microns) were the most suitable for this study. The apparatus used in conjunction with the filter membrane was a 6 centimeter size Seitz filter. A circle of # 2 Whatman paper was placed between the metal sieve and the membrane, and the entire unit was autoclaved at 120°C. for 20 minutes. The filtrations were carried out using slight suction provided by a water pump.

D - Beckman pH meter:

The Beckman pH meter, Laboratory Model G was used for all pH determinations, according to the directions located in the lid of the apparatus.

E - McIntosh and Fildes Jar:

This piece of equipment was used for two purposes.

1. For the incubation of cultures under anaerobic conditions.
2. For the incubation of cultures in an atmosphere containing 10 per cent CO_2.

1. Anaerobiosis:

The McIntosh Fildes Jar consists of a steel jar, fitted with a lid, the seal between the two being formed with the aid of

a thin film of a beeswax-vaseline mixture. The lid is equipped with two valves, two electric terminals, and on the under surface an electric light bulb surrounded by a wire net holding asbestos impregnated with pallidium black. A suction pump was used to exhaust most of the air in the jar, the vacuum resulting being replaced by hydrogen gas. The electric terminals were connected and the current switched on for 15 minutes. During this time any oxygen still remaining in the sealed jar will be reduced to water. At the end of this period the current was switched off and hydrogen was again added until atmospheric pressure was reached. All valves were then tightened, the jar being ready for incubation.

2. Carbon Dioxide 10 per cent:

The jar is sealed in the manner described above; however in this case evacuation is carried out until a vacuum of 10 mm pressure has been reached. Carbon Dioxide is then added through one of the valves, until the vacuum has been abolished. Both valves are then opened since the CO_2 being heavier than air sinks to the bottom of the jar. The jar is now ready for incubation.

F - Laboratory Flash Evaporator:

1. Description:

For evaporating the heat-sensitive solutions employed in this study the use of elevated temperatures for considerable periods of time would have led to undesirable decomposition.

Hence the Laboratory Flash Evaporator which operates under reduced pressure to lower the temperature of evaporation was used when such a procedure was required. This is shown in figure 1.

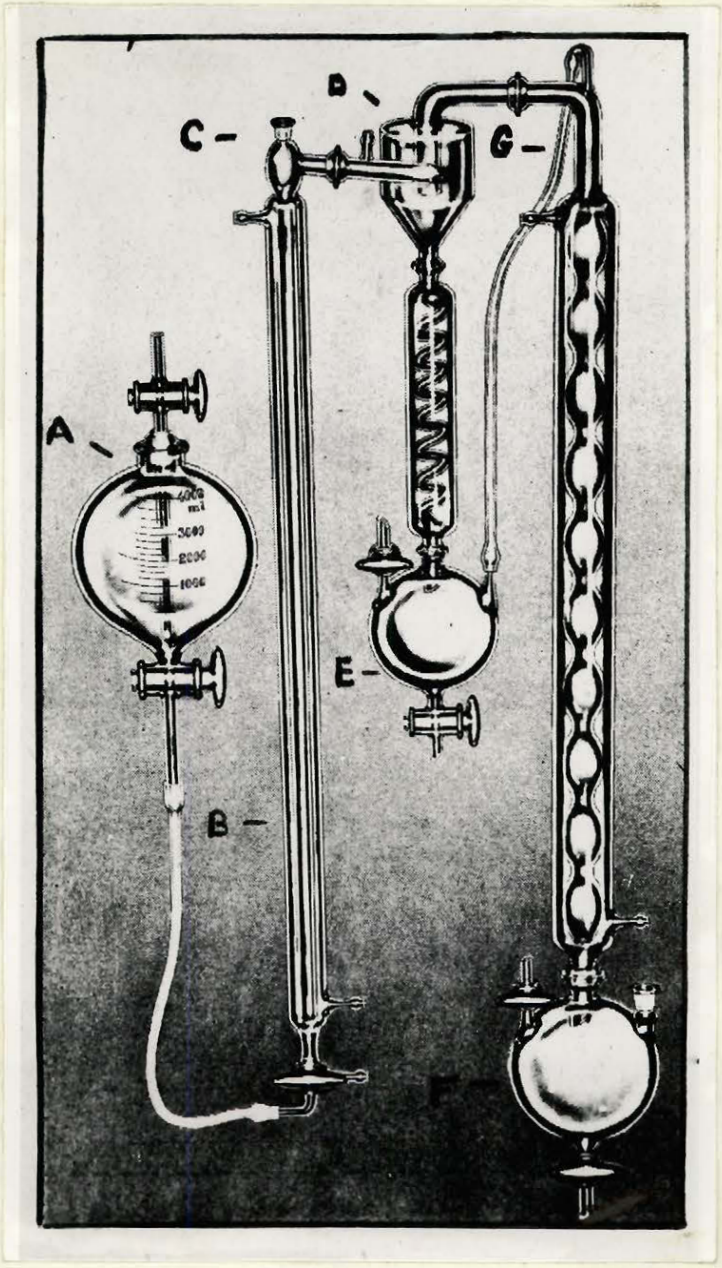

Figure 1.

The evaporator consists of a 4-litre graduated feed funnel, A, which has a constant head air inlet tube. This is joined with flexible tubing to the bottom of the steam-jacketed heating tube, B.A small rotameter in the feed line allows rapid setting of the feed rate. The vapour head of the heating tube has an opening for insertion of a thermometer, C. This head turns at a sharp angle and connects with the tangential inlet of the separator D. Provision was made for pressure measurement with a mercury manometer. The bottom outlet of the separator connects with the concentrate cooler, which joins the 2-litre concentrate receiver, E. The vapour outlet from the separator is connected through the right angle adapter to the vapour condenser which is joined in turn to the 3-litre distillate receiver, F. A stopcock controlled pressure equalizing line connects the top of the right angle adapter to the top of the concentrate receiver, G. The line connecting with the vacuum source is attached at the top of the distillate receiver. The ground glass semiball joints used to connect the various sections of the apparatus, provide a measure of structural flexibility in the unit

2. Operation:

The system is evacuated and the water and steam series are turned on. The stopcock on the constant head air inlet tube and the bottom stopcock of A are opened. The flow rate is regulated by means of the stopcock at the bottom of B. The desired feed rate can be determined by estimation from observation of the initial boiling height in the evaporator tube. Ordinarily flow

rates are selected to give a concentration of two to three parts of feed to on part of concentrate. Main points of control during operation are the temperature and pressure at the vapour head, and the feed rate. A laboratory water suction pump has sufficient capacity to maintain a satisfactory vacuum on the system.