STUDIES ON THE NUTRITION OF HEMOPHILUS INFLUENZAE

I. The Relationship between the Utilization of Coenzyme and Hemin and the Reduction of Nitrate

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The specific requirement of influenza bacilli for a heat-labile, vitamin-like substance, in addition to hemin, or X-factor, has been recognized for many years (1). Thjötta and Avery gave the name of V-factor to this substance, and in a series of studies found it to have a wide distribution both in animal and in plant tissues (2). Rivers and Poole (3) showed that a convenient differential display of these two factors could be obtained by using filtered yeast extract as V-factor and autoclaved blood as X-factor. The identity of V-factor with coenzyme was demonstrated by Lwoff and Lwoff in 1936 (4, 5). These authors suggested that the growth of *Hemophilus influenzae* be used as a quantitative measure of coenzyme by adding the test material to a medium devoid of this factor, but containing in excess all other substances necessary for growth of the microorganism. Kohn (6), Vilter, Vilter, and Spies (7), Pittman and Fraser (8), and others, have reported success in the use of the growth of *H. influenzae* and *H. parainfluenzae* as a measure of coenzyme in various materials. In all instances, turbidity has been employed as a criterion of growth of the organism, and indirectly as a measurement of coenzyme in a medium which they assumed to be adequate in all other respects for optimum growth of the bacilli. On the basis of this technique, studies on the coenzyme content in the blood of patients with pellagra (7) and of dogs with blacktongue (9) have been reported, and observations on the *in vitro* and *in vivo* synthesis of coenzyme from nicotinic acid have been made (10).

During an attempt to confirm some of these observations and to study other phases of nicotinic acid metabolism, it became apparent that additional information concerning the nutritional requirements of *H. influenzae* and *H. parainfluenzae* was needed to establish the limitation of the technique and to make certain that conclusions reached were in all instances valid. It was soon evident that the use of turbidity as a measure of the growth of the influenza bacillus was subject to many limitations. In the first place, turbidity produced by growth of *H. influenzae* is rarely great, even under optimum conditions. Under conditions imposed by coenzyme restriction changes in turbidity resulting from changes in coenzyme concentration may be almost imperceptible, although marked differences in growth may be apparent when

plating and bacterial counts are performed. Secondly, unless test substances are quite clear and free from pigment they may contribute large errors to turbidimetric measurements, and at the present time no technique of extracting coenzyme has been devised which yields a completely clear extract and which at the same time insures complete extraction. Moreover, prolonged extraction and fractionation may well result in partial destruction of coenzyme.

Successful microbiological methods have been developed recently for the quantitative determination of riboflavin (11), pantothenic acid (12), nicotinic acid and biotin (13). The emphasis placed on the determination of a stable metabolite, *i.e.*, lactic acid, in these methods, led us to search for a stable metabolic product of the influenza bacillus in the hope that the accurate measurement of such a substance could be substituted for the inaccurate measurement of turbidity as an index of growth and metabolic activity.

The ability of the influenza bacillus to reduce nitrate to nitrite has been noted consistently among its biochemical activities and was first studied in detail by Rivers (14) and Rivers and Kohn (15). In a survey of several metabolites produced by *H. influenzae* it has been found that the production of nitrite from nitrates can be used as a measurement of the metabolic activity of the bacilli and that the stability of this substance and the ease and great accuracy with which small amounts can be determined make it peculiarly applicable to a solution of the problems raised in a study of the growth requirements of this group of microorganisms. The use of this technique for the quantitative determination of coenzyme is being reported elsewhere. In this paper we wish to report studies showing the quantitative relationship between the utilization of coenzyme and hemin and the reduction of nitrate. Studies are also presented showing the influence of other substances in blood and tissue on the metabolic activity of influenza bacilli, as reflected in nitrate reduction, in the presence of optimum concentrations of hemin and coenzyme.

Materials and Methods

In an analysis of the part played by growth factors in the metabolism of *H. influenzae*, the experimental details are of great importance. Many of the equivocal and contradictory results in the literature can be explained only on the assumption that widely varying techniques were employed in attempts to elucidate similar phenomena. In some instances, large inocula have resulted in carrying over sufficient amounts of growth factors to last for several subsequent transfers. Another point contributing much confusion to an already complex picture, has been a lack of awareness of the small amounts of a given factor which may serve for the growth of *H. influenzae* in a medium in which the need for other required substances has been met. Moreover, failure to recognize the fact that X-factor is heat-labile under certain conditions may

have contributed great errors by permitting the assumption that this substance was present in excess and that increased growth of influenza bacilli was due to factors other than hemin in the materials added to the culture medium. And finally, quantitative differences in the requirement of various strains for hemin and coenzyme have not always been properly appreciated.

Source of Hemin or X-Factor.—Recrystallized, chemically pure hemin, obtained from the research laboratories of Eastman Kodak Company, was used. Similar results, however, were obtained with preparations of hemin made in our own and other laboratories. No important species differences have been observed.

Source of Coenzyme or V-Factor.—Preparations of coenzyme, or V-factor, used in this study were for the most part made in our own laboratory from yeast by the method of Williamson and Green (16). Coenzyme prepared in this manner was shown to be active in the dehydrogenation of lactate, in the presence of lactic acid dehydrogenase and methylene blue, before being used in metabolic studies with *H. influenzae.* Further characterization of our coenzyme preparations was made by spectrophotometric studies in the ultraviolet range before and after reduction of the material with sodium formaldehyde sulfoxylate. In all instances a band at 2600 Å was observed with the material in the oxidized state, and a new band at 3450 Å appeared upon reduction with sodium formaldehyde sulfoxylate. The band at 3450 Å is characteristic of reduced coenzyme (17).

Since the requirement of influenza bacilli for factor V is met by coenzyme I or by coenzyme II, and since the band of reduced coenzyme in the ultraviolet at 3450 Å is the same in either case, no individual expression of coenzyme I or II in our preparations was attempted. These studies were made in collaboration with Dr. G. I. Lavin of the Spectroscopic Laboratory of The Rockefeller Institute.

Through the kindness of Dr. B. J. Jandorf of the Department of Biological Chemistry at Harvard University Medical School, we were able to secure a sample of coenzyme with a purity of 60 per cent as determined by comparison for activity with a standard sample which showed 93 per cent theoretical value for total phosphorus and 92 per cent for reduction with dithionite. In comparison with this standard, our less highly purified preparations were shown by the growth of *H. influenzae* to have a purity ranging from 32 to 43 per cent.

A solution containing approximately 50 micrograms of the partially purified coenzyme per cc. was sterilized by Berkefeld filtration. The concentration of coenzyme in the filtrate was determined by comparison of the ability of a suitably diluted aliquot to act as V-factor with that of a standard desiccated sample of coenzyme of known purity which had been sterilized by treatment with ether. Following standardization, the filtrate was diluted to contain 4.5 micrograms of pure coenzyme per cc., and 0.5 cc. samples, accurately measured with a sterile Folin pipette, were delivered carefully into the bottoms of sterile test tubes. The material was dried *in vacuo* from the frozen state, the tubes plugged with sterile rubber stoppers and stored in a desiccator over $CaCl_2$ at 0°C. Coenzyme desiccated from the frozen state is completely and readily soluble and retains its full initial activity for several months. For use in the production of a standard growth curve with *H. influenzae* 10 cc. of

sterile distilled water were added to the dried coenzyme, care being exercised that none of the desiccated material escaped solution.

Preparation of Media.—A number of media proposed by various workers were tested. The one found best for our purposes was composed of 2.0 per cent Difco proteose-peptone, 0.6 per cent sodium chloride, and 0.2 per cent sodium or potassium nitrate. It was autoclaved for 20 minutes at 116°C. Except in those instances when the effect of hemin concentration on the growth of *H. influenzae* was being tested, 1.0 per cent autoclaved rabbit, sheep, or human blood was added to the medium after sterilization. No important differences among the bloods of various species could be determined. When blood was omitted from the medium, an autoclaved solution of hemin, as a source of X-factor, was added to make a final concentration of 0.01 mg. per cc.

Preparation of Inoculum.—The strains of *H. influenzae* used in this study were obtained from a variety of sources, the primary requirement being that the organism selected should reduce nitrate readily and consistently. The final studies reported here were for the most part carried out with two strains of *H. influenzae* each originally isolated from a case of influenzal meningitis. Standard inocula were prepared in 5 cc. of a stock broth composed of 2.0 per cent proteose-peptone, 0.6 per cent sodium chloride, 0.01 mg. per cc. of autoclaved hemin, and 0.015 micrograms per cc. of purified coenzyme. No nitrate was added in order to prevent transfer of nitrite with the inoculum. Organisms carried on "chocolate" blood agar by weekly transfers were inoculated into a tube of stock broth and incubated overnight at 37°C. After three transfers in stock broth the organisms were ready for use. When 0.1 cc. of such a culture was inoculated into 10 cc. of a medium, no multiplication of the bacilli or production of nitrite occurred unless the medium contained coenzyme.

Measurement of Nitrite Production.—For the measurement of nitrite the method proposed by Shinn (18) has been found very satisfactory. In this method the color produced by the diazotization of sulfanilamide and subsequent coupling of the diazo compound with *N*(1-naphthyl) ethylene diamine is dependent on and proportional to the nitrite present when the first two substances are added in excess. The original technique has been modified to some extent to permit its adaptation to samples containing peptone broth: —

0.1 cc. of culture, carefully measured, is added to 8.5 cc. of water, followed by 1 cc. of 0.2 per cent sulfanilamide, 0.2 cc. of 0.1 per cent *N*(1-naphthyl) ethylene diamine dihydrochloride, and 0.2 cc. of 6 N hydrochloric acid. The color develops rapidly, is maximum in 5 minutes, and shows little change after 24 hours. The color intensity is read with a photoelectric colorimeter of the Evelyn type (19) with filter No. 520.

In the determination of nitrite, the symbols and terminology proposed by Evelyn for photoelectric colorimetry have been retained. If G refers to the galvanometer reading, T to transmittance of the solution, *i.e.*, the ratio between the amounts of light transmitted by the sample and the blank, then $T = \frac{G}{100}$, when the galvanometer is adjusted to 100 with the tube containing the blank before the sample is read. For the color produced by coupling diazosulfanilamide to *N*(1-naphthyl) ethylene diamine, filter No. 520 has been found sufficiently selective to make the laws of Lambert

and Beer applicable. Hence, $C = \frac{1}{K} \times \log_{10} \times \frac{1}{T,} = \frac{1}{K} \times \log \frac{100}{G}, = 2 - \frac{\log G}{K}$. Therefore, the concentration of chromogen, $C = \frac{L}{K}$ when $L = 2 - \log G$. Here K is a constant and L is referred to as "photometric density." In these studies it has been found more convenient to use the photometric density, rather than the concentration of chromogen, as an expression of the quantity of nitrite.

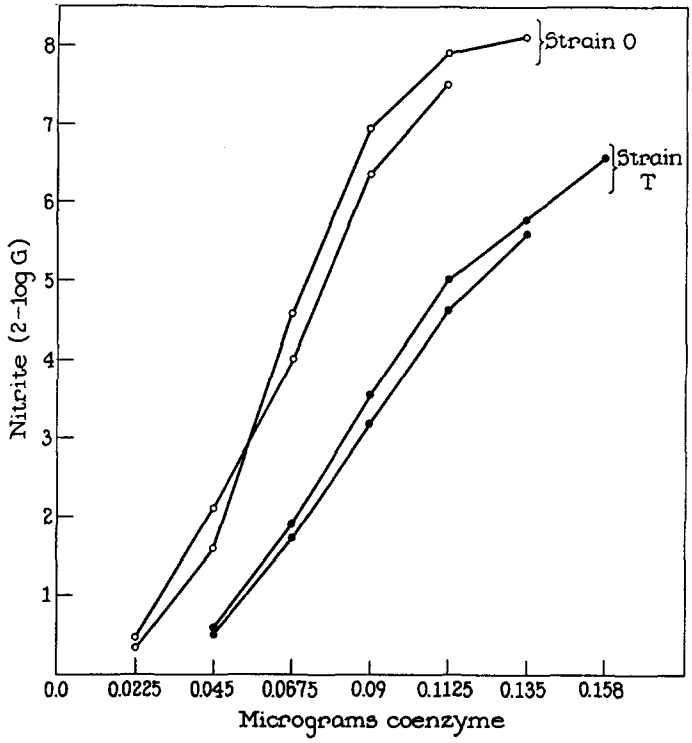
EXPERIMENTAL

In preliminary studies attempts were made to use ordinary bacteriological culture tubes for the growth of *H. influenzae*. It was soon apparent, however, that when such tubes were used the nitrite production was erratic and undependable. With media deeper than 2 cm. the amount of nitrite produced was not stoichiometrically related to coenzyme concentration. For this reason 50 cc. Erlenmeyer flasks were substituted for bacteriological culture tubes. With 50 cc. flasks containing 10 cc. of medium, consistent nitrite production, proportional to coenzyme concentration, has been achieved.

In all, five strains of *H. influenzae* have been used in this study. Although marked differences in the amount of nitrite produced were observed among different strains of influenza bacilli, the total nitrite in each instance bore a stoichiometric relationship to the concentration of coenzyme in the medium when all other factors necessary for growth were present in excess. In Text-fig. 1 it is seen that the nitrite produced by a given strain checked closely on two separate occasions. The amount of nitrite formed by strain *T*, however, was considerably less than that formed by *O*, which indicates that a given strain of *H. influenzae* may be characterized to some extent by the effectiveness with which it reduces nitrate.

Six concentrations of sterile coenzyme, 0.0, 0.0225, 0.045, 0.0675, 0.09, and 0.1125 micrograms, respectively, were added to 9 cc. of the media containing 1 mg. per cent hemin as described above, and sufficient sterile water added to bring the volume to 10 cc.; the series was set up in triplicate. The flasks were then inoculated with 0.1 cc. of a standard culture of *H. influenzae*, previously described, and incubated for 48 hours at 37°C. 0.1 cc. of culture was then added to 8.5 cc. of water and the nitrite determined as outlined above. Since the range of nitrite concentration which can be determined by diazotization and production of color by coupling is fairly narrow, and the amount of nitrite produced by *H. influenzae* is relatively great for small increments of coenzyme, it has been found best, in most instances, to limit the amount of culture taken for nitrite analysis to 0.1 cc. However, with certain strains of *H. influenzae* which reduce nitrates less effectively, 0.2 to 0.5 cc. of the culture media may be required for an accurate determination. When 1 cc. or less of the medium is taken for analysis, turbidity of the culture or test material does not interfere significantly with the photoelectric determination of the color intensity.

Heat Stability of the X-Factor Redefined.—It has been stated by various workers from time to time that X-factor in certain materials may be partially destroyed through autoclaving (20). That this destruction may be very great, even to the extent of rendering media deficient in this substance, has been brought out by quantitative studies on a comparison between the extent of growth of *H. influenzae* in media to which hemin has been added before



TEXT-FIG. 1. Relationship between the concentration of coenzyme and the production of nitrite by *H. influenzae* in a medium containing an excess of hemin.

autoclaving and autoclaved media to which X-factor, separately autoclaved, has been supplied. X-factor concentrations which, if separately autoclaved, would have been sufficient to permit maximum growth of *H. influenzae*, may, following autoclaving of X-factor and media together, fail to support visible growth of the organism. In quantitative studies on the metabolism of coenzyme by *H. influenzae* an excess of the X-factor is absolutely necessary; otherwise the growth stimulus from materials containing coenzyme may be due in part to hemin which these test substances contain.

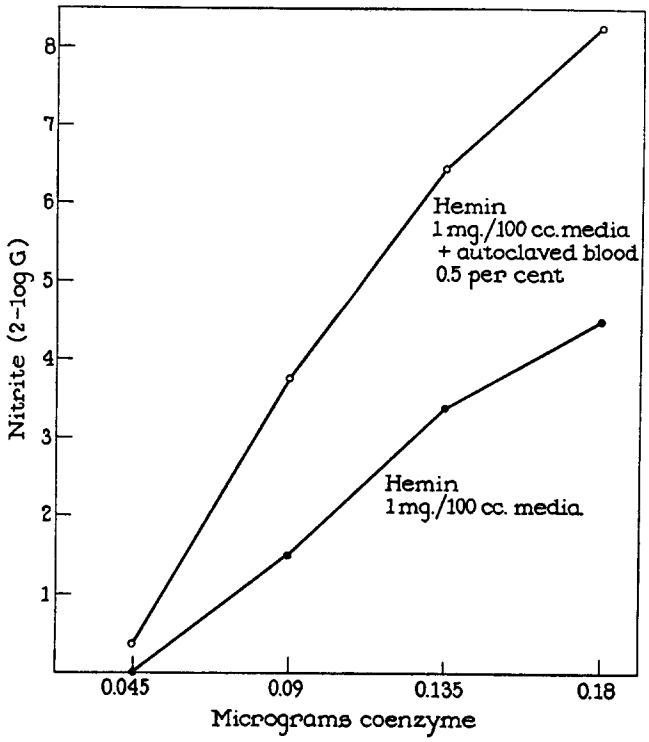
The extent to which X-factor may be destroyed by being autoclaved with the

basal medium was shown by a comparison of the growth of *H. influenzae*, in the presence of increments of coenzyme and an experimentally determined excess of autoclaved hemin, with the growth of *H. influenzae* under identical conditions, except that the hemin was autoclaved with the basal media. The growth in the second series was markedly decreased. That the decreased growth was caused by a lack of sufficient X-factor was shown by the fact that the basal media were again rendered adequate for optimum growth by the addition of hemin which had been autoclaved separately. In a number of reported studies in which the growth of *H. influenzae* has been used as a measure of coenzyme concentration, these factors have not been controlled. Consequently it is possible that any observed stimulation was due to additional X-factor added with the coenzyme test material, and not to the coenzyme, or V-factor, alone. This criticism may be particularly leveled at those reports in which a proteose-peptone medium, without the addition of sufficient X-factor, has been used in the quantitative assay of coenzyme. For example, media prepared according to Vilter, Vilter, and Spies (7) has, in our hands, consistently failed to grow *H. influenzae* when highly purified coenzyme preparations were added to supply V-factor. Growth is obtained, however, when hemin is added subsequently, or when a blood extract, prepared according to the technique of these workers, is used as a coenzyme test substance. In the latter instance sufficient hemin is carried over in the acid extract to permit growth in the presence of the coenzyme which the extract contains. However, when test substances which contain little hemin are being assayed, the factor limiting growth of *H. influenzae* is no longer due to coenzyme, but to the variable quantity of hemin which may be present.

Additional Factors in Blood Which Stimulate the Growth of H. influenzae.— The development of a more exact quantitative method for the determination of the metabolic activity of *H. influenzae* has made possible a study of the effect of various nutrient substances on the growth of this group of organisms. That there are additional factors in blood, other than hemin and coenzyme, which enhance the growth of *H. influenzae* is evident in the data portrayed in Text-fig. 2.

To duplicate flasks of 10 cc. of basal media were added an experimentally determined excess of hemin (1 mg. per 100 cc.) and increments of coenzyme from 0.0 to 0.1125 micrograms. These were inoculated with a standard strain of *H. influenzae*, prepared in the manner previously described and incubated for 48 hours at 37°C. Nitrite determinations were made and the results have been plotted in Text-fig. 2 against increments of coenzyme. Another series of flasks containing 0.5 per cent autoclaved rabbit blood, in addition to the constituents of the basal medium, were set up, and hemin to make a final concentration of 1.0 mg. per cent was added. Increments of coenzyme, as described in the first series, were added, and the flasks inoculated with 0.1 cc. of a standard culture of *H. influenzae* and incubated for 48 hours.

In Text-fig. 2 the results of nitrite reduction *vs.* increments of coenzyme are compared with the series in which hemin, but no blood, had been added to the proteose-peptone medium. In all instances a definite and measurable increase in nitrate reduction was noted when blood was added to the basal medium. The addition of more than 0.5 per cent of blood, however, failed to increase stimulation. It would appear, therefore, on the basis of these studies, that



TEXT-FIG. 2. Production of nitrite by *H. influenzae* in a medium containing in crements of coenzyme and an excess of hemin, and with and without the addition o 0.5 per cent blood.

additional factors, either organic, inorganic, or both, in addition to hemin and coenzyme, may enhance the growth of *H. influenzae* in the basal medium, and that these are maximally supplied by autoclaved blood when it is added in a concentration of 0.5 per cent.

Influence of Known Growth Factors on the Metabolism and Growth of H. influenzae.—In order to use the metabolic activity of the influenza bacillus as an index of coenzyme concentration it is important to know what effect may be expected from an increased concentration of other known growth factors

which may be added in varying amounts as contaminants in coenzyme test materials. In Table I are listed a large number of substances which have been added to blood-enriched, proteose-peptone media inoculated with *H. influenzae*. In no instance has any significant difference been noted in the amount of nitrite produced between the control cultures grown in basal media with added hemin and coenzyme and those cultures containing large amounts of added growth factors in addition to the constituents of the control media. Moreover, a number of amino acids, added separately and in combination, produced

TABLE I

Effect of Added Nutrient Materials on the Growth and Metabolism of H. influenzae in a Basal Medium Containing an Excess of Hemin and Coenzyme

[Substance	Concentration per cc. of medium	Effect on growth as reflected in nitrite production	Substance	Concentration per cc. of medium	Effect on growth as reflected in nitrite production
	micrograms			*milligrams*	
Pantothenic acid	0.05	None	β-Alanine	0.5	None
Nicotinic acid	0.05	"	Inositol	0.1	"
Nicotinamide	0.05	"	Ascorbic acid	0.05	"
Pyridoxin	0.05	"	Cytochrome *c*	0.05	"
Thiamin	0.05	"	Ergothioneine	0.025	"
Biotin	0.002	"	Tryptophane	0.05	"
Choline	0.05	"	Arginine	0.05	"
Folic acid	0.05	"	Amino acid mixture:		
Paraminobenzoic acid	0.05	"	Methionine 0.06 Tryptophane 0.06 Histidine 0.06 Leucine 0.20 Glycine 0.02 Glutamic acid 0.04 Arginine 0.06	0.50	"
Riboflavin	0.05	"			
Riboflavin adenine dinucleotide	0.05	"			
Riboflavin phosphate	0.05	"			
Thiamin pyrophosphate	0.05	"			

no significant alteration of growth or metabolic activity in this medium. These are likewise recorded in Table I.

The fact that influenza bacilli do not use nicotinic acid as such, but require its physiologically active form, coenzyme, led us to try the activated forms of riboflavin and thiamin, *i.e.*, riboflavin adenine-dinucleotide, riboflavin phosphate, and thiamin pyrophosphate. Riboflavin adenine-dinucleotide was prepared from yeast by the method of Warburg and Christian (21), riboflavin phosphate was synthesized by the method of Kuhn, Rudy, and Weygand (22), and thiamin pyrophosphate was synthesized by the method of Weijlard and Tauber (23). No stimulation by any of these substances to the growth or metabolic activity of *H. influenzae* could be detected when they were added to

the blood-enriched proteose-peptone medium. It cannot be concluded from these results, however, that these substances are not required by the influenza bacillus, since the heat stability of riboflavin adenine-dinucleotide, riboflavin phosphate, and thiamin pyrophosphate, unlike coenzyme, would insure a certain concentration of these catalysts in the blood-enriched basal medium. It can only be stated that if these factors are active in promoting the growth of *H. influenzae* they are present in excess in the basal medium, since added amounts produced no detectable stimulus to growth or metabolic activity over that observed in control cultures.

DISCUSSION

In the foregoing studies we have attempted to define more carefully the conditions under which the growth and consequent metabolic activity of *H. influenzae* may be correlated with the total coenzyme, or V-factor, content of the medium. It is obvious that unless all specific requirements for the growth of the influenza bacillus are fully met, a quantitative relationship between metabolic activity and coenzyme content of the medium cannot be expected. Moreover, it is essential that substances not specifically required for growth, but which influence its extent, must likewise be supplied in excess, otherwise they may have a marked stimulating effect on the degree of growth when added with materials which are being tested for coenzyme activity.

The difficulties previously encountered in the measurement of the growth of *H. influenzae* have been overcome by a technique which substitutes for the measurement of turbidity the quantitative determination of the nitrite produced by these organisms in a medium to which nitrate has been added. The production of nitrite has been shown to be a consistent measure of the metabolic activity of the influenza bacillus, and, when other requirements of the organism are met in excess, bears a consistent relationship to the concentration of coenzyme in the medium. In these studies the nitrate reduction technique has been used in an attempt to define the optimum growth requirements of *H. influenzae*, which, in turn, are to form the basis of further studies on the nutrition and metabolism of this organism.

When optimum amounts of hemin and coenzyme are present, additional requirements and growth-modifying factors appear to be supplied in excess when 0.5 per cent blood is added to a proteose-peptone medium. This is shown by the fact that no appreciable alteration in growth or metabolic activity of *H. influenzae* can be demonstrated when an increased concentration of various factors which are known to enhance bacterial growth are added to a blood-enriched proteose-peptone medium. It is essential in those instances in which hemin is added as the main source of X-factor that the hemin be autoclaved separately, otherwise sufficient destruction may result to render the medium deficient in this substance.

From these studies it appears that extensive information concerning factors affecting the growth and metabolism of a test organism must be had before it may be used accurately as a specific biological reagent for microbiological assay. Not only is it essential to know the specific factors required for growth of the organism and the optimum concentration of these factors, but information must likewise be had concerning those substances which, although not essential for the organism, may, nevertheless, affect the extent of its growth.

CONCLUSIONS

The metabolic activity of *H. influenzae* can be followed quantitatively by measurement of the nitrite produced in a medium containing 0.2 per cent potassium or sodium nitrate.

When X-factor, or hemin, and other specific substances required for the optimum growth of *H. influenzae*, are present in excess, the nitrite produced by this organism is quantitatively related to the concentration of V-factor, or total coenzyme. This quantitative relationship has been demonstrated for five strains of *H. influenzae*.

It has been shown that various media, which in the past have been used for the determination of coenzyme by growth of *H. influenzae*, have in many instances been deficient in X-factor and that this substance rather than coenzyme has been the specific factor limiting growth.

When 0.5 per cent blood is added to a basal proteose-peptone medium the specific requirements for optimum growth and metabolic activity of *H. influenzae*, other than coenzyme, are met, and a large number of specific biocatalysts and nutritive substances added to this medium are without effect in stimulating further growth.

The foregoing studies have formed the basis for a quantitative method for the determination of total coenzyme in blood and tissue. This method is being described elsewhere.

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