CODEHYDROGENASE I AND OTHER PYRIDINIUM COMPOUNDS AS V-FACTOR FOR HEMOPHILUS INFLUENZAE AND H. PARAINFLUENZAE

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Nutritional requirement of complete coenzymes in place of, or in preference to, the vitamin moieties (e.g., nicotinamide, thiamine) of their respective molecules is apparently not common even among parasitic organisms. Lwoff and Lwoff (1936a, b, 1937a, b) demonstrated that preparations of codehydrogenases I and II satisfy the V-factor requirement of *Hemophilus parainfluenzae* whereas nicotinamide does not. Their results were confirmed by a number of investigators, chiefly in the course of developing and using assay methods for experimental and clinical studies, using both *Hemophilus parainfluenzae* (Kohn *et al.* 1938–1940, Pittman and Fraser 1940, Dann, Handler *et al.* 1941, 1942, and others) and *Hemophilus influenzae* (Vilter *et al.* 1939, 1940, Hoagland *et al.* 1942, 1943, and others). It was further shown that these organisms are able to utilize nicotinamide riboside in place of V-factor although rather poorly as compared with codehydrogenase I (Schlenk and Gingrich, 1942). In this connection it is interesting to mention the fact that the growth response of *Streptococcus salivarius* is better in the presence of cocarboxylase than with thiamine (Niven and Smiley, 1943).

The fact that the codehydrogenases can be substituted for V-factor has led to the use of hemophilic bacteria for attempts to develop assay methods for the coenzymes in tissue fluids, extracts and excretions. Although the results are somewhat divergent the findings indicate generally that the V-factor content of blood is not related to the intake of nicotinamide nor to deficiency thereof, but that it is reduced in liver and muscle of dogs suffering from blacktongue. This agrees with the results of Axelrod *et al.* (1939, 1941) for the codehydrogenase I content of tissues as determined by the fermentation test. That the microbiological assay methods have not come into general use is to be explained partly by the fact that blood determinations fail to yield a ready means of diagnosing nicotinamide deficiency and also to the variability of the growth response of the organisms and the difficulty of interpreting results in absolute terms unless relatively pure preparations of codehydrogenase are used for reference.

Our attention has been directed particularly to the questions as to (1) which compounds satisfy the V-factor requirement and (2) how they may be utilized in comparison to codehydrogenase I. In attempting to reach reasonably tenable conclusions we have considered the use of pure compounds of primary importance and are confident of having satisfied the requirements in this respect (Schlenk, 1942a). The indications of the results relative to the function of V-factor and

[1] The authors acknowledge the technical assistance of Mrs. C. H. Cox, Jr.

to the biosynthesis of codehydrogenase I from its structural units are especially interesting to us. And, although the development of a bioassay method has not been our primary concern, the relation of the results to this problem have been borne in mind.

MATERIAL AND METHODS

The compounds which were examined for growth-promoting properties (as substituted for V-factor) for *H. influenzae* and *H. parainfluenzae* are described as follows. Formulae are shown in table 1.

Codehydrogenase I (cozymase) was prepared according to previously described methods (Schlenk and Günther, 1940 and Schlenk, 1942a). The preparation was standardized and purity was determined by the fermentation test (Myrbäck, 1933) and by spectrographic measurement of the absorption coefficient at 340 mμ upon reduction with hydrosulfite: $E = 5.5 \times 10^6 \left[\frac{cm^2}{mole}\right]$ (Hilger quartz spectrograph, model E 498). Observed values of catalytic hydrogenation according to Warburg and Christian (1936) were 101 and 97.5 cu mm H_2; calculated, 101 cu mm H_2.

Elementary analysis: $C_{21}H_{27}O_{14}N_7P_2 \cdot 2H_2O$ (699.2)
Calculated. 36.04% C, 4.46% H, 14.01% N, 8.87% P;
Found. 35.79% C, 4.23% H, 13.53% N, 8.76% P.

Dihydro-codehydrogenase I was prepared from codehydrogenase I as the sodium salt, as described by Ohlmeyer (1938). The degree of purity (>90%) was tested as described above for codehydrogenase I. A trace of alkali was added to the solutions to prevent spontaneous oxidation to codehydrogenase I which occurs slowly at pH 6 to 7.

Acid-treated codehydrogenase I was prepared by dissolving dihydro-codehydrogenase I in a small amount of 0.1 N HCl and allowing it to remain at room temperature for 3 to 5 minutes before neutralization with an equal amount of 0.1 NaOH. With some samples containing traces of codehydrogenase I the latter was first destroyed by dissolving the sample material in 0.1 N NaOH. After 2 hours at room temperature, all codehydrogenase is destroyed under these conditions whereas dihydro-codehydrogenase I remains unchanged. Subsequent acid treatment as described above yielded the derivative which is inactive in the fermentation test.

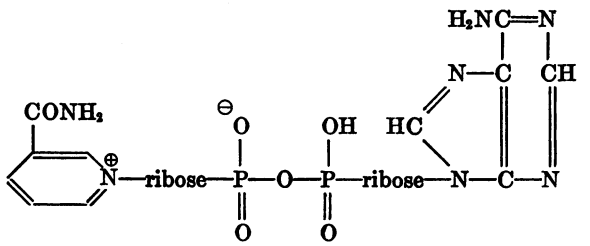
The sample of codehydrogenase II was 50 per cent pure as determined spectrophotometrically. The fermentation test indicated a 2 per cent content of codehydrogenase I. (The authors are indebted to Dr. Erwin Haas for this preparation.)

Desamino-codehydrogenase I was prepared by treating codehydrogenase I with KNO_2 in 2N acetic acid and the resulting product was isolated as previously described (Schlenk, Hellström and v. Euler, 1938). This artificial derivative shows the same optical properties (ultraviolet absorption) as codehydrogenase I. In the fermentation test, however, its activity is only 35 to 40 per cent as compared with the natural coenzyme.

TABLE 1

Formulae of pyridinium compounds tested

1.



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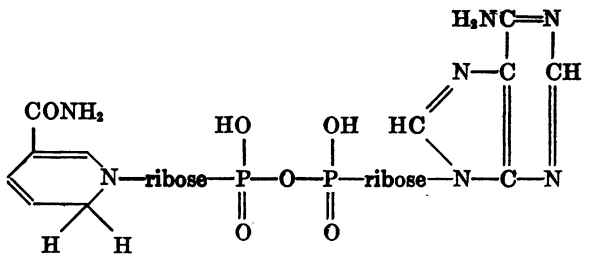

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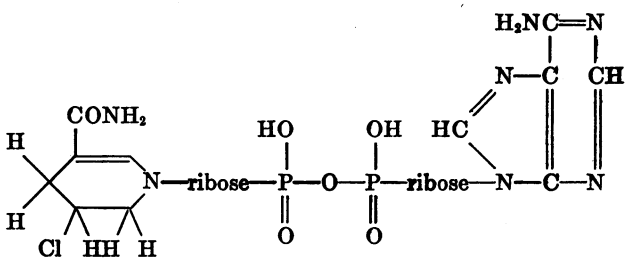

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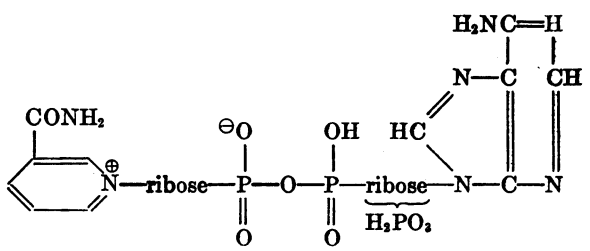

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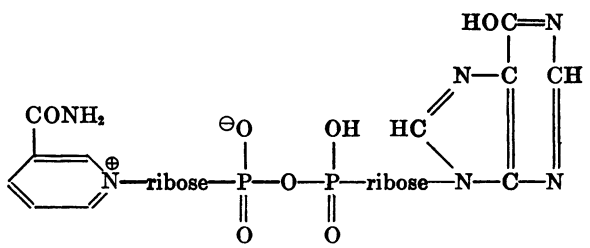

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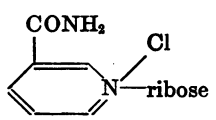

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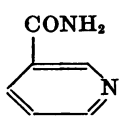

1, Codehydrogenase I; 2, dihydro-codehydrogenase I; 3, acid treated dihydro-codehydrogenase I; 4, codehydrogenase II; 5, desamino-codehydrogenase I; 6, nicotinamide riboside; 7, nicotinamide.

Nicotinamide riboside was obtained by splitting codehydrogenase I with a phosphatase preparation from sweet almonds. The isolation and purification of this compound has recently been described (Schlenk, 1942b, 1943). Two preparations were used, both over 90 per cent pure as determined by spectrographic analysis and by pentose and nicotinamide determinations. They were inactive in the fermentation test, indicating the absence of codehydrogenase I.

Nicotinamide, d-ribose and adenine were obtained commercially and adenosine and adenylic acid were prepared according to the methods given in the literature.

Solutions of these compounds were passed through Seitz filters for sterilization. HCl was added to 0.01 N to prevent deterioration of codehydrogenase I, desamino-codehydrogenase I and nicotinamide riboside.

The organisms used in this work are rough variants although one of them (strain O) had been smooth or intermediate and one (strain B) had been mucoid (as defined by Pittman, 1931, and Chandler *et al.* 1939) for a short period of time. They are:

Hemophilus parainfluenzae,

4101, National Collection of Type Cultures, Lister Institute (obtained from Dr. H. I. Kohn of Duke University). 7901, American Type Culture Collection.

Hemophilus influenzae,

8149, American Type Culture Collection.

V, obtained from Dr. S. P. Vilter, University of Cincinnati.

O, obtained from Dr. C. L. Hoagland, Rockefeller Institute.

T, obtained from Dr. C. L. Hoagland, Rockefeller Institute.

B, isolated locally from a fatal case of meningitis.

Stock cultures were maintained on a medium prepared from North's gelatin agar (Difco) and hemoglobin (Difco) supplemented to 10 per cent with filtered yeast extract prepared from 1 gram dried yeast in 50 ml water at 90°C for 10 minutes, pH 7.8. Cultures were transferred weekly, incubated for 24 hours, and stored at room temperature.

The medium used for testing the several compounds for growth-promoting properties consisted of Bacto peptone 1 per cent, glucose 0.1 per cent, NaCl 0.6 per cent, $NaNO_3$ 0.2 per cent, pH adjusted to 7.8, autoclaved. To one liter of medium was added 10 ml of 0.5M phosphate buffer of pH 7.8 and, for experiments with *H. influenze,* 10 ml of 0.1 per cent hemin solution, each sterilized separately in the autoclave, before distributing in sterile apparatus to 50 ml Erlenmeyer flasks in 9 ml portions. Supplements of yeast extract, blood extract, adenosine, etc., were used only for special purposes as described below.

The procedure of setting up the tests became regular after a number of trials and it proved advantageous to adhere quite rigidly to the routine. The medium was distributed in sterile flasks as soon as it was cooled sufficiently to be handled, placed in the refrigerator immediately and stored for two days before the compound to be tested was added. Sterile water in suitable quantities for dilution of the test substance was also stored in the refrigerator. Following addition of the substance in a volume of 1 ml with a minimum exposure to room temperature

the flasks were again stored in the refrigerator for 4 to 6 hours or more until the inoculum was ready. The cultures were incubated at 37° for 18 hours.

For preparation of the inoculum, codehydrogenase I was added to a concentration of 2×10^{-5} γ-mol (0.01326 γ) per ml of a flask of medium similar to that used in the test except that it contained no $NaNO_3$. Within 4 to 6 hours incubation, following inoculation from an actively growing culture (6 to 8 hours) on stock medium, this culture was usually in the late logarithmic or early stationary phase and therefore suitable for use as inoculum for the test. To each flask (except the control for sterility of the test substance) of a given experiment was added one drop of the inoculum culture.

Results were observed by (1) reading turbidity visually, (2) measuring turbidity with a photometer and (3) quantitative determination of the nitrite content of the cultures. Visual reading of turbidity was particularly advantageous for the lower ranges of titrations where slight blanching of the medium and minimal growth precluded photometric comparison of the density of the cultures. Nephelometric determinations were made with the electrophotometer of Fisher Scientific Co., which had been modified to accomodate 25 mm culture tubes and to measure 10 ml quantities by shading both absorption cells. In practise, the cultures in the 50 ml flasks were shaken well to remove any growth adherent to the glass and poured into the calibrated 25 × 150 mm tubes. Suitable checks were made to ascertain that all growth had been transferred from the flasks to the tubes. Samples were then taken for the nitrite determinations. The method of Shinn (1941) adapted by Hoagland, Ward, Gilder and Shank (1942) was used for the determination of nitrite content of the cultures. Samples of 0.1 ml were taken for the determinations of cultures of the strains of *H. influenzae* and samples of 1 ml for those of *H. parainfluenzae*, as the latter produced considerably less nitrite. The reagents and procedure used were the same as those described by the authors mentioned above except that the results are expressed here as the amount of $NaNO_2$ per ml of culture.

RESULTS

Minimum concentrations of the compounds required for detectable growth

Data collected from 36 titrations (in which, first, the several compounds were titrated with a given organism and, secondly, a given compound was titrated with the several organisms) as well as a number of special tests serve as a basis for the following discussion. The minimum concentrations of compounds supporting growth were determined as well as possible in the absence of a definite end point. Although it was necessary to rely on visual reading of turbidity for most of these data, careful reading and repeated tests yielded fairly consistent findings. The results as shown in table 2 represent the concentrations which regularly supported growth. Occasionally, growth was detected in lower concentrations but such data are unsatisfactory as a basis for comparison because of the irregularity of occurrence.

Referring to the tabulated results it is evident that codehydrogenase I is equally effective, on the basis of minimum concentration for detectable growth

(2×10^{-6} γ-mol, or 0.001326 γ, per ml), for all strains except 7901. Codehydrogenase I, it should be stated, yielded the most consistent results throughout all the experiments. This applies not only to the concentration required for minimal growth but also to the growth curve of the titration. And, of the compounds tested, only nicotinamide riboside promoted growth in lower concentration. Subculture of all strains through 35 transfers was accomplished without difficulty on a peptone-salt-glucose agar (plus hemin for *H. influenzae*) containing 1×10^{-3} γ-mol codehydrogenase I per ml of medium.

Hemophilus parainfluenzae 7901 had a long lag period and grew poorly in the test medium even in the presence of great excess of coenzyme. In view of the fact that its growth on the stock medium is abundant, the inference is clear that the test medium is deficient for this organism. The data for strain 7901 with all the compounds tested therefore merely serve to emphasize the fact that the results depend on the extent to which the test medium is complete for the test organism.

Dihydro-codehydrogenase I supported growth of all the strains down to the same concentration as did codehydrogenase I. The difference of $3 \times$ and 2×10^{-6} γ-mol per ml for detectable growth of strain B with these two compounds can hardly be considered significant. Occasionally the reduced coenzyme promoted growth rather poorly, with turbidity observed only in concentrations of 7×10^{-5} γ-mol per ml or greater, whereas in the same experiment codehydrogenase I supported growth as usual. The relative stabilities of the two compounds is not the factor responsible for this variation since dihydro-codehydrogenase I is more stable at an alkaline pH than is codehydrogenase I. One sample of the dihydro-codehydrogenase I which yielded good results probably contained a trace of the oxidized coenzyme. We do not, however, consider this a valid reason for rejecting the data for it does not seem possible that a trace of codehydrogenase I could lead to a completely false titration as illustrated in figure 1, of the reduced coenzyme. It is possible that some condition or other of the test may affect growth with dihydro-codehydrogenase I which apparently does not affect growth with codehydrogenase I.

Since our first report on the activity of pyridinium compounds, we have tested acid-treated dihydro-codehydrogenase I more thoroughly. In five tests, each with several strains, the limit of growth was reached at concentrations varying from 5×10^{-4} γ-mol per ml to 1×10^{-2} γ-mol per ml, with most of the determinations at 7×10^{-4} γ-mol. Since the ratios of these results to those for the corresponding samples of dihydro-codehydrogenase I are 1:333 or less it is apparent that the presence of 0.3 per cent or less of codehydrogenase I in the preparations could account for the slight growth which occurred in the higher range of the titrations. However, one sample which was titrated with all the strains promoted growth down to $6 \times$ and 7×10^{-4} γ-mol per ml whereas the tests with the corresponding sample of dihydro-codehydrogenase I yielded growth down to $3 \times$ and 5×10^{-6} γ-mol per ml. This latter result is explained by a contamination of the preparation with approximately 2 per cent of the oxidized coenzyme (which is not inactivated by treat-

ment with acid). Slight activity in the fermentation test demonstrated the presence of this amount of active coenzyme. Our previously reported utilization of acid treated dihydro-codehydrogenase I in concentrations down to 5×10^{-5} γ-mol per ml is therefore rather explained in the same manner. In the light of this analysis we feel justified in concluding that the acid treatment of dihydro-codehydrogenase I renders it inactive for growth of *H. influenzae* and *H. parainfluenzae* as well as it does for fermentation by isolated yeast enzymes.

The data for codehydrogenase II are rather limited, three titrations with 4101 and two each with the other strains. Whether the minimum concentrations of 7, 5 and 3×10^{-6} γ-mol per ml are significantly different for the various strains or from 2×10^{-6} γ-mol for codehydrogenase I can therefore hardly be determined with the data at hand. Certainly it can be stated, however, that codehydrogenase II supports minimal growth of these organisms in a concentration approximating that of codehydrogenase I. Some indication as to the manner

TABLE 2

Concentrations of pyridinium compounds required for detectable growth of Hemophilus influenzae and H. parainfluenzae

STRAIN	CODEHYDROGENASE I	DIHYDRO-CODEHYDROGENASE I	CODEHYDROGENASE II	DESAMINO-CODEHYDROGENASE I	NICOTINAMIDE RIBOSIDE
	γ-mol $\times 10^{-6}$ per ml of medium				
H. parainfluenzae strain 4101	2	2	5	5	0.5
H. parainfluenzae strain 7901	5	5	10	7	0.5
H. influenzae strain 8149	2	2	5	7	0.5
H. influenzae strain V	2	2	7	7	0.5
H. influenzae strain O	2	2	3	5	0.5
H. influenzae strain T	2	2	3	3	0.5
H. influenzae strain B	2	3	3	30	500* 1

* For explanation see text.

in which this compound is utilized may be found by comparing these results with the growth curve of the titration.

The results of tests with nicotinamide riboside substituted for V-factor are interesting in several respects. The minimum concentration of 0.5×10^{-6} γ-mol per ml as given in table 2 is lower than that of any other compound for minimal growth of all the strains except B—i.e., one-fourth the concentration of codehydrogenase I required for the same effect. In fact, the titrations which were carried one step farther to 0.1×10^{-6} γ-mol per ml showed evidence of growth in this concentration in one-third of the trials. This latter concentration is the same as the final concentration of codehydrogenase I in the test medium which is transferred with the inoculum, that is, on the assumption that all of the coenzyme of the inoculum culture remained available as such at the end of the 4- to 6-hour incubation. If we assume half the original amount of coenzyme in the inoculum to be available at the time it is used to inoculate the test medium, the final concentration in the latter would be

0.05 $\times$ 10^{-6} γ-mol per ml which is one-tenth of the lowest concentration we have considered significant. Since smaller inocula yield less consistent results and the coenzyme is for the most part contained within the living cells we have thus arrived close to the limit of the test. The significant fact here is that nicotinamide riboside promotes growth of *H. influenzae* and *H. parainfluenzae* in a concentration well below (less than one-fourth) that of codehydrogenase I. Furthermore the riboside is the simplest compound which can be substituted for the V-factor required by these organisms, for nicotinamide added up to 1γ-mol per ml of medium failed to support growth of any of the strains. Final proof of the substitution of nicotinamide riboside for V-factor was demonstrated by continued subculture through 28 transfers of all the strains except B (*vide infra*) on peptone- salt-glucose agar (plus hemin for *H. influenzae*) containing 1 $\times$ 10^{-3} γ-mol of the pure compound per ml of medium.

The behavior of strain B of *H. influenzae* merits special consideration with respect to its utilization of nicotinamide riboside and desamino-codehydrogenase I. This strain was isolated locally from a fatal case of meningitis in January, 1943, and yielded results similar to those of other strains in two titrations with codehydrogenase I. When first titrated with nicotinamide riboside, however, it grew poorly in the higher range (1 $\times$ 10^{-2} and 1 $\times$ 10^{-3} γ-mol per ml) and not at all below 5 $\times$ 10^{-4} γ-mol per ml whereas other strains showed evidence of growth down to 1 $\times$ 10^{-6} (no further dilutions tested). At this time strain B appeared to be a smooth but not a mucoid culture, lacking iridescence. With three attempts it failed to grow on the second transfer on peptone-salt-glucose-hemin agar containing 1 $\times$ 10^{-3} γ-mol riboside per ml although it grew well on the same medium with codehydrogenase I. One month later, following five transfers and without apparent change in character of growth or microscopic morphology, this organism showed minimal growth with 2 $\times$ 10^{-6} γ-mol riboside per ml. Following this titration and starting with the same lot of nicotinamide riboside agar used previously, the strain was carried through 25 successive transfers with abundant growth. With two further titrations (April and May) this strain grew with 1 $\times$ 10^{-6} γ-mol riboside per ml but not at 5 $\times$ 10^{-7} whereas the others did. The growth of the organism appears now to be intermediate between smooth and rough.

Two titrations of desamino-codehydrogenase I were performed with *H. influenzae* strain B (March and May), both with the same result of growth detected down to 30 $\times$ 10^{-6} γ-mol per ml. Growth of the other strains was observed down to 3 to 7 $\times$ 10^{-6} γ-mol per ml. Since these figures are based on only two to four tests for each of the organisms we are not inclined to attach any significance to the differences between strains except that of strain B. Except for the latter strain, this derivative appears to be utilized slightly less efficiently in comparison with codehydrogenase I as judged by the minimum concentration required for detecting growth.

Titration growth curves

The concentrations in the titrations usually ranged from 1 $\times$ 10^{-2} to 1 $\times$ 10^{-6} γ-mol per ml or less. Tests with a given preparation and with the several strains

were designed to show any possible differential utilization by the various organisms and those with the different compounds and a given organism were expected to demonstrate the relative efficiency of the compounds. Figure 1 illustrates the results of one of the latter experiments with strain 4101, the densities being represented as the photometer readings (logarithmic scale). It is evident that codehydrogenase I and the reduced coenzyme are about equally effective throughout the titration and are more efficient than all the other pyridinium compounds which had any activity whatever. The curve for desamino-codehydrogenase I parallels that for the natural coenzymes at a reduced effectiveness. On the basis of equal culture densities the relative efficiency of the desamino derivative is

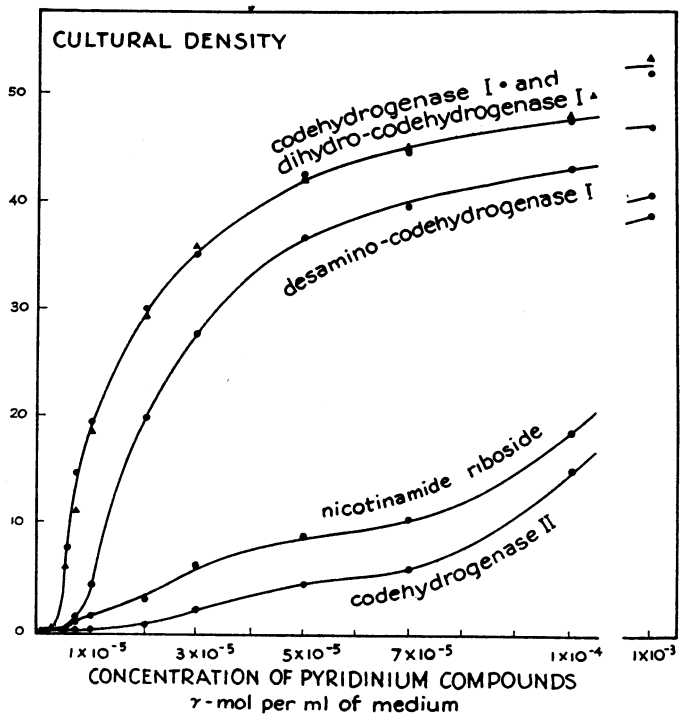

FIG. 1. TITRATION OF PYRIDINIUM COMPOUNDS WITH HEMOPHILUS PARAINFLUENZAE STRAIN 4101

about 60 per cent, although it varies somewhat depending on the segments of the curves used for comparison.

Nicotinamide riboside and codehydrogenase II evidently are utilized in a manner quite different from the other compounds. The former has an activity of about 4.5 per cent at 1×10^{-3} and 10 per cent at 1×10^{-4} and 5×10^{-5} γ-mol per ml as compared with the cultural densities produced with the complete coenzyme. Such a low efficiency is the more remarkable in view of the fact that this compound is effective in promoting minimal growth of these organisms in even lower concentrations than codehydrogenase I. Codehydrogenase II, the triphosphopyridine nucleotide, is even less efficient, 4 to 8 per cent, and the

growth curve is of the same type as that for the nicotinamide riboside. Similar results demonstrating the relative activity as V-factor and indicating the manner of utilization of these pyridinium compounds were obtained with all the strains of *H. influenzae* and *H. parainfluenzae* listed above.

The range of variability in our experience with these titrations has led us to regard the data as approximations rather than determinations and to consider the estimated relative efficiencies of the several compounds accordingly. Concerning the variation above a given point in the same experiment, for example, we obtained densities of strain 4101 in six cultures containing 1×10^{-3} γ-mol nicotinamide riboside per ml ranging from 41.2 to 49.5 with an average of 46.0 $\pm \sigma 3.4$ and at a concentration of 1×10^{-4}, densities of 19.5 to 25.0, average 21.05 $\pm$ 1.8. As to values observed from one experiment to another the same strain with 1×10^{-3} γ-mol codehydrogenase I per ml yielded readings of 39.5 to 68.0 with an average of 53.0 $\pm$ 10.1, and at 3×10^{-5} γ-mol per ml, 23.0 to 44.5, average 33.4 $\pm$ 6.9. The slope of the curves varied considerably in different experiments.

Reduction of nitrate

The two strains of *H. parainfluenzae* reduced nitrate very poorly or not at all in the test medium. The test for nitrite in cultures of strain 4101 was frequently negative but occasionally determinations yielded the equivalent of 4 γ $NaNO_2$ per ml. With the addition of autoclaved yeast extract to the same medium this organism produced 35$\gamma NaNO_2$ per ml in the presence of 1×10^{-2} γ-mol codehydrogenase I per ml. Strain 7901 did not reduce nitrate except in the presence of extract of blood or yeast and then only very slightly.

All five strains of *H. influenzae* reduced nitrate well, although the nitrite determinations varied considerably in different experiments with the same test substances and organisms. The amount of nitrite was roughly proportional to the amount of codehydrogenase I or other substituents for V-factor, but gross irregularities frequently occurred. The presence of nitrite was usually detected in cultures with codehydrogenase I in a concentration of 2×10^{-5} γ-mol per ml and occasionally with 7 or 5×10^{-6} γ-mol per ml. Some of the data (table 3) taken from one experiment illustrate the comparative curves of nitrate reduction and of growth in relation to the concentration of coenzyme.

Nitrate reduction is evidently more sensitive to cultural conditions than is the growth of the organism. For example, a small amount of HCl added to a concentration of 0.001 N in the medium with an excess of codehydrogenase I depressed nitrate reduction to a far greater extent than it affected the growth of the organism (see table 3). This effect was evidently not due directly to a change in hydrogen ion concentration in the medium as whatever change occurred was within 0.1 pH unit as measured by the glass electrode. Another factor influencing the reaction is the type of supplement added to the medium, whether for assay or for X-factor. In an attempt to determine the suitability of various media we tested autoclaved hemin (10γ per ml of medium) and extract of autoclaved beef blood (equivalent to 1 per cent blood in the medium) with the results as

shown in table 4. With almost identical growth response the wide variation in nitrate reduction, depending on the supplement for X-factor, is remarkable. Curiously enough, autoclaved yeast extract added to the medium depressed nitrite production by half in one experiment with strain T although its effect, if any, was generally the reverse.

TABLE 3

Comparison of culture density and nitrite content of H. influenzae with varying concentrations of codehydrogenase I

γ-MOL CODEHYDROGENASE I PER ML MEDIUM	STRAINS OF H. INFLUENZAE							
	8149		V		O		T	
	Density	Nitrite	Density	Nitrite	Density	Nitrite	Density	Nitrite
1.0*	56	19	54	44	56	180	66	203
0.1	58	590	65	1377	75	316	70	322
0.01*	50	0	52	44	60	177	68	44
1×10^{-3}	55	400	66	1371	72	635	65	506
5×10^{-4}	52	564	66	438	74	415	61	415
1×10^{-4}	49	332	55	700	66	435	57	349
7×10^{-5}	23	277	46	374	54	267	37	132
3×10^{-5}	14	25	24	170	36	88	19	55
2×10^{-5}	11	15	17	97	21	88	11	15
1×10^{-5}	9	0	8	75	8	100	8	103
7×10^{-6}	9	0	6	64	7	26	6	58
5×10^{-6}	5	0	4	15	2	0	3	0
3×10^{-6}	1	0	0	0	0	0	3	0

* Solution of codehydrogenase I in 0.01 N HCl, therefore HCl added to the medium to 0.001 N.

TABLE 4

Influence of supplement for X-factor on nitrate reduction

γ-MOL CODE-HYDROGENASE I PER ML MEDIUM	H. INFLUENZAE, STRAIN 8149					
	Hemin		Blood extract		Hemin + blood extract	
	Density	Nitrite	Density	Nitrite	Density	Nitrite
1×10^{-3}	47	740	44	205	45	935
1×10^{-4}	31	272	31	225	28	435
1×10^{-5}	1	0	3	0	1	15

Factors influencing growth

From a theoretical viewpoint it seemed desirable to determine whether other fractions of the coenzyme molecule would influence growth in the presence of nicotinamide riboside. Accordingly, we added adenosine to the medium (1×10^{-2} γ-mol per ml) with 1×10^{-3}, 1×10^{-4} and 1×10^{-5} and 3×10^{-6} γ-mol of riboside per ml and found no significant difference as compared with duplicates without adenosine with any of the seven strains. Nor did the addition of autoclaved yeast extract improve the growth of strains 4101 and T with the riboside.

From a practical point of view it is essential to control the factors influencing growth if any of the organisms are to be used for assay of codehydrogenase I. Aeration is certainly a factor as demonstrated by Pittman (1935) and by the more consistent results of culturing the organisms in shallow medium as observed by Hoagland *et al.* (1942) and in our own work. It may be questioned, however, whether a depth of 0.5 to 1 cm of medium takes care of this factor adequately. In this connection it may also be mentioned that on one occasion when the test medium was stored in the room for one week it failed to support growth of any of the organisms even in the presence of excess coenzyme. Addition of riboflavin did not correct the defect. The frequent depression of growth, by the addition of HCl (0.001 N) too small in amount to change the pH of the buffered medium significantly, has been referred to previously (table 3). Finally, the unexpected variation encountered in this work has indicated to us that either there are unknown factors influencing the growth of these organisms or the known factors are not satisfactorily controlled by the conditions of culture—or both.

DISCUSSION

The natural coenzyme, codehydrogenase I and its reduced derivative, dihyrocodehydrogenase I, promote growth of *Hemophilus influenzae* and *H. parainfluenzae* more effectively than any other compound tested. Taking this into consideration, with the fact that it is also present in tissues in greater concentration than any other pyridinium compound, except possibly nicotinamide, it is very definitely indicated therefore that the V-factor required by these organisms is identified as codehydrogenase I. It seems likely that the coenzyme diffuses into the bacterial cell and is utilized as such, for nicotinamide riboside + adenosine is far less effective. The fact that the riboside promotes minimal growth in somewhat lower concentrations than codehydrogenase I might be explained on the basis of differential rates of diffusion. Nicotinamide riboside probably represents the limit of adaptability of the organisms in using simpler compounds as V-factor since it is utilized much less efficiently in suitable concentrations. However, the fact that it is utilized at all by these organisms may be an indication that organisms with lower requirements synthesize the coenzyme by linking nicotinamide and ribose as one of the primary steps.

Codehydrogenase II is utilized so poorly and in a manner so closely parallel to that of nicotinamide riboside as to lead one to speculate that the compound is ineffective as such and that it is split to yield the riboside to account for what little activity it does have. Desamino-codehydrogenase I, on the other hand, is probably utilized without change. Although we have no information indicating that it is not converted to the proper coenzyme it seems likely that it could be utilized as the desamino derivative which has an activity of 40 per cent as compared with codehydrogenase I in the fermentation test with yeast apoenzyme and substrate. The difference between 40 per cent activity in the fermentation test and 60 per cent activity in cultures of *H. influenzae* and *H. parainfluenzae* is probably due to the difference in the apoenzymes. Certainly the evidence is

contrary to the presumption that the organisms split the compound to yield nicotinamide riboside for it is far more active than the latter.

The variability of the nitrite content of the cultures renders this metabolite unsuitable for assay methods—at least under the conditions of our experiments. Considering the importance of physical factors of the medium one is led to suspect that even nutrient supplements influence both nitrite production and growth by altering physical conditions. The results do, however, raise interesting questions as to the actual conditions which favor nitrate reduction and what the function of the reaction may be in the metabolism of the organisms. The difference between strains of *Hemophilus influenzae* and *H. parainfluenzae* in the reduction of nitrate is marked and of regular occurrence. Relatively little is known about the nitrate-reducing enzymes of these organisms. However, the cyanide sensitivity of the nitrate reductase described by Quastel (1932) and by Yamagata (1938) for *Escherichia coli* indicates a hemin-like prosthetic group for the enzyme. This would fit nicely into the picture of X-factor as required by strains of hemophilic bacteria which reduce nitrate and the lack of such requirement of strains which do not.

For the assay of codehydrogenase I with any of the strains listed above it must be admitted that the methods now in use permit only approximations rather than any appreciable degree of precision. Frequently one may obtain results which might be reproduced with some degree of accuracy for a period of time but even then it may be questioned whether the standards for reference yield reliable results. Since autoclaved yeast extract influences both growth and reduction of nitrite it is evident that relatively crude or impure preparations of coenzyme are likely to yield false points of reference. Similarly, extracts of materials assayed can affect the test so as to indicate results far too high or too low. Furthermore, the error due to the presence of nicotinamide riboside, codehydrogenase II or other substituents for V-factor might be large or small depending on the relative concentrations of these compounds. The problem of developing a method of assay with a degree of accuracy approaching those for nicotinamide therefore revolves about finding an organism with relatively simple requirements and the ability to control the test medium with respect to the requirements. If the results are to be expressed as anything more definite than V-factor or codehydrogenase "equivalents" the test organism will have to be more specific in its requirement.

SUMMARY

1. The minimum concentration of codehydrogenase I and the reduced coenzyme, dihydro-codehydrogenase I, which promotes growth of *Hemophilus influenzae* and *Hemophilus parainfluenzae* is 2×10^{-6} γ-mol (0.001326γ) per ml. They are the most efficient compounds as substituents for V-factor.

2. Nicotinamide riboside supports minimal growth of these organisms in concentrations of 5×10^{-7} γ-mol per ml or lower. However, as it is far less efficient than the complete coenzyme (even in the presence of adenosine) and is present in animal tissues only in insignificant amounts, it is relatively unimportant

as V-factor in the natural habitat of hemophilic bacteria. It may be considered to be the limit of adaptability of the organisms in synthesizing the complete coenzyme when it is not available.

3. Codehydrogenase II is less efficient than nicotinamide riboside and the curve of growth in relation to concentration parallels that of the latter compound.

4. Desamino-codehydrogenase I, an artificial derivative, satisfies the V-factor requirement of *H. influenzae* and *H. parainfluenzae* with an efficiency of about 60 per cent as compared with codehydrogenase I. Since the curve of growth in relation to concentration of the compound parallels that of the natural coenzyme, it is probably utilized by the organisms without change or may be converted to the proper coenzyme by restoration of the amino group.

5. Factors influencing growth of the organisms and nitrate reduction in relation to the bioassay of codehydrogenase I, or V-factor, are discussed.

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