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FURTHER STUDIES ON FACTOR W

By

Douglas Van Anden Frost

A Thesis Submitted for the Degree of Master of Arts

UNIVERSITY OF WISCONSIN

1938

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By D. V. FROST and C. A. ELVEHJEM

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison*)

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FURTHER STUDIES ON FACTOR W*

BY D. V. FROST† AND C. A. ELVEHJEM

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison*)

(Received for publication, August 5, 1937)

In a previous paper Elvehjem, Koehn, and Oleson (1) presented evidence for the existence of a water-soluble vitamin present in liver extract and yeast which was distinct from the known vitamins. Rats placed on a purified diet in which vitamin B_1 was supplied in crystalline form, vitamins B_4 and B_6 from white corn, and flavin and the antipellagra factor in concentrates prepared from liver extract failed to grow until the entire liver extract or yeast was added. This factor could be separated from liver extract by treating a concentrated aqueous solution of the powder with large volumes of a mixture of alcohol and ether. It was tentatively designated the alcohol-ether precipitate factor. Since our knowledge of this factor grew out of our original studies on the antipellagra factor, and since liver extract was used as a source of the factor, there has been some tendency to confuse this factor with the antipellagra factor. It should be emphasized that the new factor is separate and distinct from the antipellagra vitamin. The alcohol-ether precipitate fraction was completely inactive in the prevention of pellagra-like lesions in chicks (2). In order to obviate this difficulty and to eliminate the term alcohol-ether precipitate factor, which is both long and misleading, we have substituted temporarily the simple term, factor W.

In the work which has been published flavin was added to the basal ration as a concentrate prepared from liver extract and fed

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

A preliminary report of this work was presented before the Thirty-first meeting of the American Society of Biological Chemists at Memphis, April 21–24, 1937.

† Eli Lilly and Company Fellow.

in amounts equivalent to 2 per cent of the liver extract. Certain investigators have criticized this procedure on the basis that the concentrate might not supply sufficient flavin and that the precipitate fraction merely supplied additional amounts of this essential factor. In this paper we wish to present further evidence that the profound growth-stimulating property of the alcohol-ether precipitate fraction is due to a factor distinct from flavin and to give additional information about the properties of this factor.

EXPERIMENTAL

We have continued to use Ration K_{12} described previously (1) as the basal ration. It has the following composition.

	gm.
Reprecipitated skim milk casein	18
White corn	12
Dextrin	58
Butter fat	5
Salts I	4
Cod liver oil	2
	micrograms
Crystalline vitamin B_1	120

When crystalline vitamin B_1 became available at a reasonable price, the amount added was increased to 120 micrograms per 100 gm. of ration in order to eliminate any possible deficiency of this factor when the food consumption was decreased. We have used both the Winthrop and Merck products. In some series Labco casein was substituted for the casein prepared in our laboratory, with equal results.

This ration supplies sufficient amounts of vitamins B_1, B_4, and B_6, but is low in flavin, factor W, and probably the antipellagra factor. Halliday and Evans (3) have recently reported that they obtained growth and cure of florid dermatitis in rats on a low vitamin B_6 diet with the alcohol-ether precipitate fraction from whole liver and concluded, therefore, that vitamin B_6 is adsorbed during the precipitation and that the precipitate does not carry a new factor. One of us (C.A.E.) has pointed out in an addendum to their paper that their results do not conflict with our conclusions. As far as Ration K_{12} is concerned, it is only necessary to point out that it carries 12 per cent white corn, and most workers are agreed that this level would supply adequate vitamin B_6.

Also rice bran, which is a good source of vitamin B_6, gives a poor response when fed as a source of factor W.

In our earlier work concentrates of the antipellagra factor were added to the basal ration. Further studies have shown that identical results are obtained with and without this supplement. Some workers (4) have concluded that rats do not require the antipellagra vitamin in any significant amount. We do not have

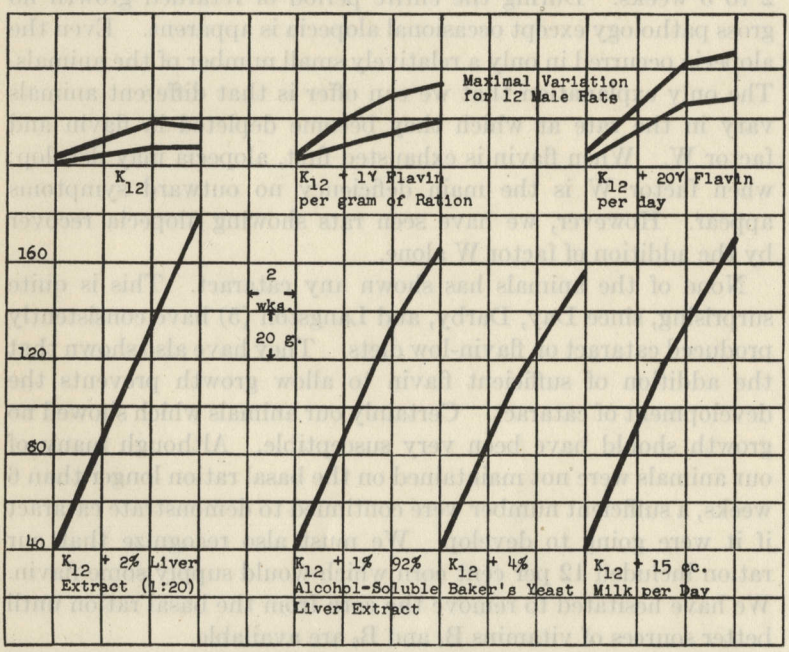

CHART I. Growth curves for rats on basal Ration K_{12}, basal plus flavin, and basal plus supplements containing both flavin and factor W.

enough data to allow a definite conclusion and can only suggest that our basal ration contains sufficient amounts of this factor or that the experimental period was not long enough to induce a deficiency.

The only remaining deficiencies in Ration K_{12}, therefore, are flavin and factor W. If flavin were the only deficiency, normal growth should result when pure flavin is added. The results obtained by the addition of pure flavin are given in Chart I. All

rats used in this work were taken from our regular stock colony and placed on experiment when 21 to 28 days of age.

Rats placed on Ration K_{12} with no supplement show practically no growth. If they do grow, it is only during the 1st week or 2, and after that the weight either remains constant or declines slightly. The animals rarely die before 6 weeks, but if they are continued on the ration, they generally succumb after another 2 to 5 weeks. During the entire period of retarded growth no gross pathology except occasional alopecia is apparent. Even the alopecia occurred in only a relatively small number of the animals. The only explanation that we can offer is that different animals vary in the rate at which they become depleted in flavin and factor W. When flavin is exhausted first, alopecia may develop; when factor W is the main deficiency no outward symptoms appear. However, we have seen rats showing alopecia recover by the addition of factor W alone.

None of the animals has shown any cataract. This is quite surprising, since Day, Darby, and Langston (5) have consistently produced cataract on flavin-low diets. They have also shown that the addition of sufficient flavin to allow growth prevents the development of cataract. Certainly our animals which showed no growth should have been very susceptible. Although many of our animals were not maintained on the basal ration longer than 6 weeks, a sufficient number were continued to demonstrate cataract if it were going to develop. We must also recognize that our ration included 12 per cent corn which would supply some flavin. We have hesitated to remove the corn from the basal ration until better sources of vitamins B_4 and B_6 are available.

When Ration K_{12} was supplemented with 1 microgram of pure flavin[1] per gm. of ration, a small but definite growth response resulted. The maximal variation in the growth of twelve male rats receiving this level of flavin is compared with the basal ration in Chart I. The growth response obtained when each rat received 20 micrograms of flavin per day is also shown. The growth is only slightly better and cannot compare with the growth obtained in the rats receiving supplements containing both flavin

[1] We are indebted to Dr. S. Lepkovsky, University of California, and Dr. J. W. Hart of the Winthrop Chemical Company, Inc., for generous supplies of flavin.

and factor W. The average weekly gain over the 6 week period never exceeded 10 gm., which is less than one-third the normal rate obtained in the latter groups. The addition of 40 micrograms daily gave no better growth than 20 micrograms daily. The inability of flavin to produce normal growth unless accompanied by factor W is more clearly evident from the results obtained when flavin and concentrates of factor W free of flavin were fed alone and in combination.

In the previous work regular commercial liver extracts (Lilly, Wilson, and Abbott) were used as starting material for the preparation of the alcohol-ether precipitate. About a year ago Dr. C. E. Graham of The Wilson Laboratories sent us several liver fractions which were by-products in the manufacture of the pernicious anemia fraction. One of the fractions was found to be highly active as a source of factor W. The growth curves in Chart I show that Ration K_{12} supplemented with 1 per cent of this material gave a growth response equal to that obtained by an addition of 2 per cent liver extract, 4 per cent bakers' yeast, or 15 cc. of milk daily. The active fraction represents the filtrate remaining after the precipitation of the pernicious anemia factor with 92 per cent alcohol. It is a dark, viscous material containing 60 per cent solids. 1 part of this concentrate is equal to 200 parts of the original fresh liver. The material as obtained reduces methylene blue and gives a strong reduction with Fehling's solution.

Since the 92 per cent alcohol-soluble liver fraction contained considerable protein, no complete fractionations could be made with such reagents as fullers' earth, mercury salts, or phosphotungstic acid. Attempts were made, therefore, to obtain a more homogeneous and easily workable concentrate by extracting with various solvents. Amyl alcohol, ether, and dioxane were completely ineffective, but acidified aqueous acetone yielded a clear brown solution containing all the activity and practically no protein. Extraction with dry acetone (Sullivan's method) removed no activity.

The solution was acidified because we had found that much of the factor W in the alcohol-ether precipitate from regular liver extract was closely associated with protein. Continuous dialysis of this product removed less than one-half of the total activity in

several days. When the precipitate fraction was first treated with weak acid, a much larger portion of the activity was recovered in the dialysate.

Method

1 kilo of 92 per cent alcohol-soluble liver extract was placed in 1 liter of water containing 5 cc. of 1:1 HCl, and 9 liters of acetone were added with constant shaking. The acetone fraction was

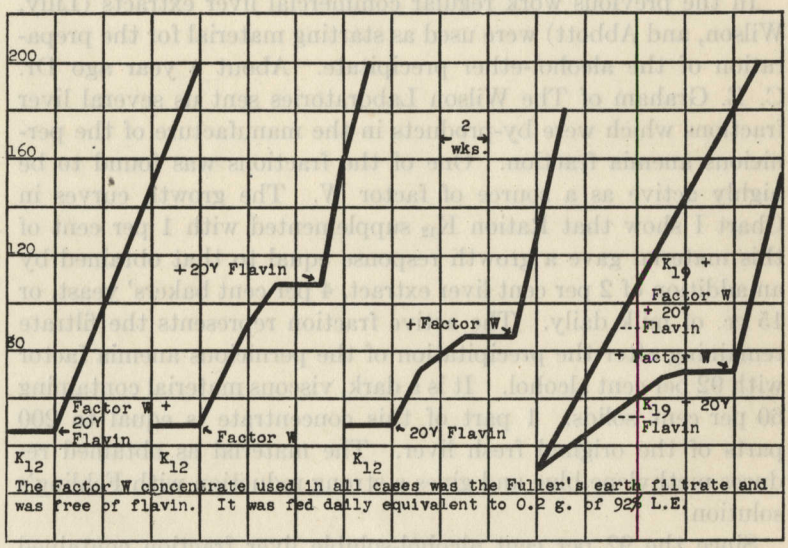

CHART II. Growth curves showing typical responses of rats on basal rations K_{12} and K_{19} to flavin alone, concentrates of factor W alone, and the combination of the two.

decanted off and the residue treated in the same way with a mixture of 200 cc. of water, 5 cc. of 1:1 HCl, and 3 liters of acetone. After six more similar extractions the residue contained very little activity. The acetone was recovered from the combined extracts by ordinary distillation and the last traces were removed by vacuum distillation. When the dark, aqueous solution was diluted with water to 5 liters, considerable denatured protein separated out. This was readily removed by filtering on filtercel. The clear brown solution was quantitatively freed of flavin by

adsorption on 500 gm. of fullers' earth at a pH of about 3. In some preparations the fullers' earth adsorption was repeated but it was found unnecessary if the pH was kept at 3 or below.

Typical growth curves of rats given this concentrate of factor W with and without flavin are shown in Chart II. Flavin was fed at a level of 20 micrograms per rat per day and the factor W concentrate at a level equivalent to 0.2 gm. of the original material per day. The response when both were added to the diet of rats maintained on Ration K_{12} until growth had ceased was immediate and profound. When factor W was added alone, there was good growth for about 2 weeks, after which the growth curve reached a plateau. If flavin was added at this point, there was immediate resumption of growth, which continued at a rapid rate for some time. Responses of 50 gm. in 1 week have been very common. An actual increase in weight of 15 gm. may be observed 24 hours after the addition is made. When flavin was added alone the growth stimulation was not so great nor so prolonged as with factor W alone. The addition of factor W, after growth had ceased on flavin, produced the characteristic growth response. These results demonstrate very clearly the importance of supplying ample amounts of factor W as well as flavin when any of the other vitamin B factors are being studied.

Some attempts have been made to aggravate the factor W deficiency through the use of a more highly synthetic ration in the hope of producing a more definite pathology. We used a ration (K_{19}) made up as follows: dextrin 76, casein (Labco) 18, Salts I 4, cottonseed oil 2, and vitamin B_1 200 micrograms. 2 drops daily of wheat germ oil containing 2 per cent percomorph oil (Mead's) supplied 60 international units of vitamin A and 9 international units of vitamin D. Rats raised on this regimen plus 20 micrograms of flavin daily ceased to grow after 3 weeks and maintained themselves at about 70 gm. for considerable periods (Chart II). These animals showed no dermatitis or other gross deficiency symptoms. The addition of the factor W concentrate after several weeks on flavin alone again gave the phenomenal growth response. An average growth curve for two rats receiving flavin and factor W from the beginning is included for comparison.

Consumption records were kept on these animals for the first 5

weeks on the respective regimens. The average daily intake of the two animals maintained on Ration K_{19} plus 20 micrograms of flavin per day was 3.5 gm. The average intake of the two litter mates raised on Ration K_{19} plus 20 micrograms of flavin plus adequate factor W was 8.0 gm.

The addition of cod liver oil directly to the ration in place of the wheat germ and percomorph oil gave identical results. This ration has been designated Ration K_{20} and has been used for the assay of certain of the concentrates. The majority of the assays have been made on Ration K_{17} which is identical with Ration K_{12} except 1 microgram of flavin is added per gm. of ration. At first the concentrates to be tested were added directly to the basal ration but as the preparations became more highly purified they were fed to the rats in small porcelain cups. Very little difficulty has been encountered in getting the animals to consume the preparations. The activity is based on the gain in weight over a 5 week period.

Many procedures have been tried for the separation and concentration of factor W; however, only the more significant results will be outlined.

Adsorption on Charcoal—In the previous paper (1) we reported that this factor was adsorbed on charcoal but that difficulty was encountered in the removal of the active material from the charcoal. Further attempts to elute the factor with alcoholic pyridine or ammonium hydroxide were entirely unsuccessful.

When work on the aqueous liver extract was discontinued in favor of fractionation of the 92 per cent alcohol-soluble material, attempts to use charcoal for adsorption of factor W were continued. Difficulty was now encountered in recovery of the active material because both the filtrate and the charcoal proved inactive. Since the supply of the charcoal used in the first work was exhausted, we thought that the variable results might be due to the use of a different charcoal. Nuchar and norit A (Pfanstiehl) were then tried, with the result that no activity could be recovered. The charcoal and filtrates were fed separately and together at very high levels but none showed any appreciable growth-stimulating effect (see Chart III). We thought that factor W might be held so effectively by these activated charcoals that the animal could not remove the vitamins, but elution with

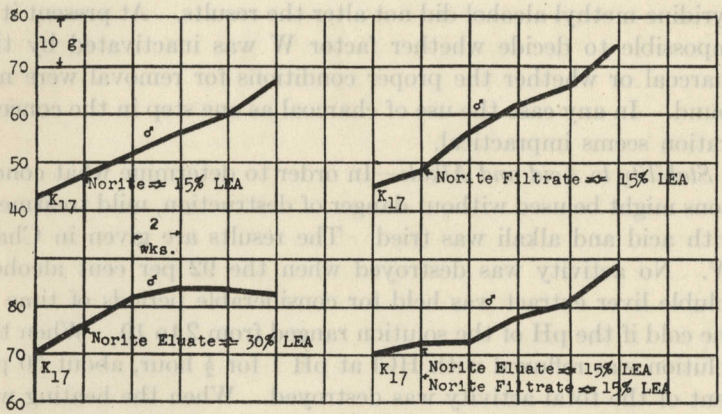

CHART III. Results showing the inability to recover the activity of factor W from concentrates treated with charcoal.

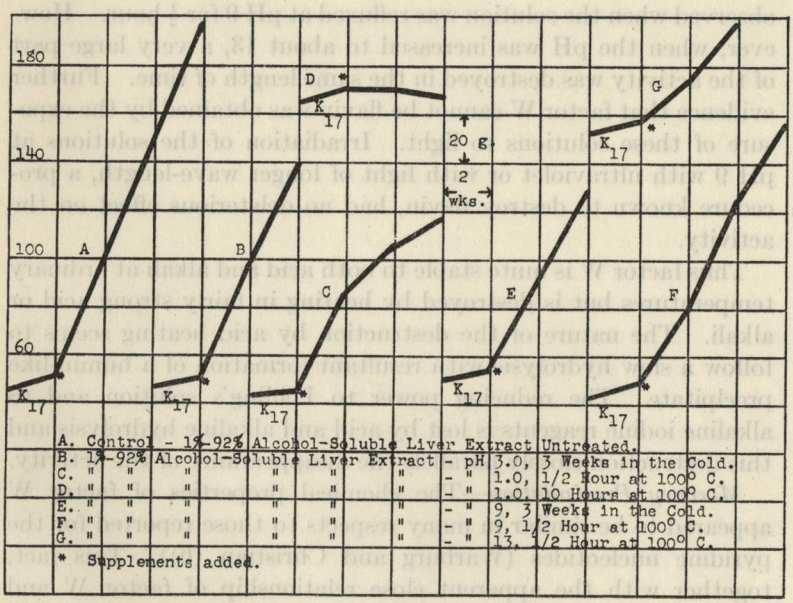

CHART IV. Growth curves for rats on basal Ration K_{17} plus supplements of 1 to 92 per cent alcohol-soluble liver extract previously subjected to acid or alkali treatments, as indicated in Curves B to G.

pyridine-methyl alcohol did not alter the results. At present it is impossible to decide whether factor W was inactivated by the charcoal or whether the proper conditions for removal were not found. In any case the use of charcoal as one step in the concentration seems impractical.

Stability to Acid and Alkali—In order to determine what conditions might be used without danger of destruction, mild treatment with acid and alkali was tried. The results are given in Chart IV. No activity was destroyed when the 92 per cent alcohol-soluble liver extract was held for considerable periods of time in the cold if the pH of the solution ranged from 2 to 10. When the solution was refluxed with HCl at pH 1 for ½ hour, about 50 per cent of the total activity was destroyed. When the heating was continued for 10 hours, all activity disappeared. The same results were obtained by refluxing at pH 3 for 3 hours. No detectable decrease in activity as measured with Ration K_{17} was observed when the solution was refluxed at pH 9 for ½ hour. However, when the pH was increased to about 13, a very large part of the activity was destroyed in the same length of time. Further evidence that factor W cannot be flavin was obtained by the exposure of these solutions to light. Irradiation of the solutions at pH 9 with ultraviolet or with light of longer wave-length, a procedure known to destroy flavin, had no deleterious effect on the activity.

Thus factor W is quite stable to both acid and alkali at ordinary temperatures but is destroyed by heating in fairly strong acid or alkali. The nature of the destruction by acid heating seems to follow a slow hydrolysis with resultant formation of a humin-like precipitate. The reducing power to Fehling's solution and to alkaline iodine reagents is lost by acid and alkaline hydrolysis and this destruction closely parallels the disappearance of the activity.

Mercury Precipitation—The chemical properties of factor W appeared to be similar in many respects to those reported for the pyridine nucleotides (Warburg and Christian (6)). This fact, together with the apparent close relationship of factor W and flavin in the biological response, led us to investigate the possible identity of factor W and the various coferments. Since we had obtained factor W in an aqueous acetone solution, it was possible to proceed directly according to the method developed by War-

burg for the precipitation of pyridine nucleotides and other purine derivatives from an acetone extract of red blood cells.

The flavin-free fullers' earth filtrate was treated with 600 gm. of $Hg(OAc)_2$ dissolved in a minimum of warm water and the pH adjusted to 7 for maximum precipitation. The mercury precipitate was allowed to settle in the cold for 24 hours and centrifuged. The precipitate was washed twice with 1 per cent $Hg(OAc)_2$. The precipitate was then suspended in about 1 liter of water and treated with H_2S with shaking until precipitation of the mercury was complete. After centrifuging, the HgS was suspended in water

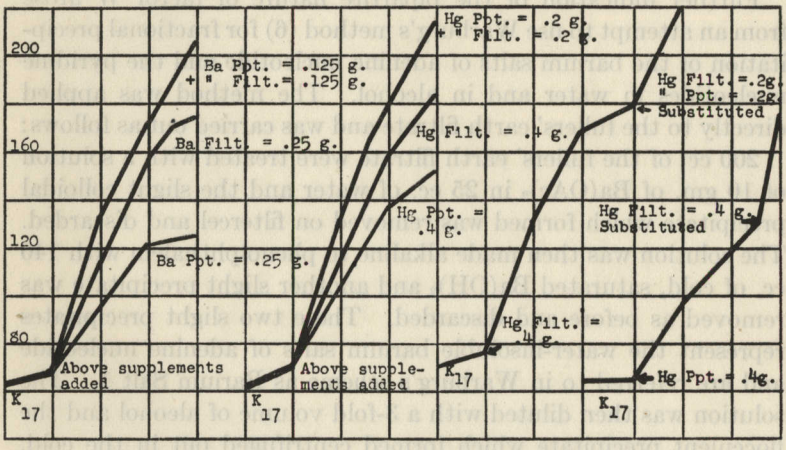

CHART V. Growth curves showing the activity of mercury and barium fractions.

and air was passed through the suspension for 2 hours. This effects elution of much of the active material from the highly adsorbent HgS. The combined filtrates were concentrated *in vacuo* and tested for mercury. This fraction was designated the mercury precipitate fraction. The filtrate from the original mercury precipitation was freed of mercury with H_2S and concentrated *in vacuo* to remove the occluded H_2S and was designated the mercury filtrate fraction.

The activity of these preparations when added to Ration K_{17} is shown in Chart V. The mercury precipitate fed at a level equivalent to 0.4 gm. of the original material daily gave a growth

response of 15 to 18 gm. per week. This was not an optimum response but we felt that this decreased activity might be due to a natural loss during the process of fractionation. However, the addition of higher levels did not give greater increments in growth. Actually, the mercury filtrate gave a better growth response when fed at an equivalent level. The combination of the two fractions fed at one-half the level of the individual supplements gave still better growth. These assays have been repeated several times with identical results. This result gave the first suggestion that we might be dealing with more than one factor.

Further indication of the bipartite nature of factor W arose from an attempt to use Warburg's method (6) for fractional precipitation of the barium salts of adenine nucleotide and the pyridine nucleotides in water and in alcohol. The method was applied directly to the fullers' earth filtrate and was carried out as follows:

200 cc. of the fullers' earth filtrate were treated with a solution of 10 gm. of $Ba(OAc)_2$ in 25 cc. of water and the slight colloidal precipitate which formed was removed on filtercel and discarded. The solution was then made alkaline to phenolphthalein with 140 cc. of cold, saturated $Ba(OH)_2$ and another slight precipitate was removed as before and discarded. These two slight precipitates represent the water-insoluble barium salts of adenine nucleotide and are referred to in Warburg's scheme as Barium Salt 1. The solution was then diluted with a 3-fold volume of alcohol and the flocculent precipitate which formed centrifuged out in the cold. The precipitate was not washed, but was dissolved directly in 100 cc. of water. Barium was removed quantitatively from the aqueous solution with a minimum of sulfuric acid and the concentrate was made to pH 5 for assay. This barium precipitate fraction corresponds to the Barium Salt 2 of Warburg and represents the barium salt of triphosphopyridine nucleotide (coenzyme). The alcoholic filtrate, which in the procedure of Warburg contains the barium salt of diphosphopyridine nucleotide (cozymase) and is called the Barium Salt 3, was also freed of barium with sulfuric acid and prepared for assay.

Repeated assays of the fractions singly and in combination at half levels showed somewhat greater growth for the combined fractions than for either fraction alone (Chart V).

The apparent indispensability of both the mercury precipitate

fraction and the mercury filtrate fraction for optimum growth received further confirmation from the fact that the diminishing growth of animals maintained on either fraction alone for 5 to 6 weeks was immediately stimulated by supplementation of the other. The marked increase in growth rate induced by substitution of the mercury filtrate fraction for the mercury precipitate fraction might logically be due to the greater potency of the filtrate fraction. However, growth was also enhanced when the precipitate fraction was supplied together with the filtrate fraction to animals maintained on the filtrate fraction until growth had slowed. This was true even though the level at which the mercury filtrate had been fed was halved at the time of introducing the mercury precipitate fraction. No growth stimulation occurred when the mercury precipitate was substituted for the mercury filtrate. This may merely indicate that no storage of the factor carried by the mercury filtrate occurs under these conditions, but that storage of the factor carried by the precipitate does occur.

These findings necessarily changed the plan of our investigation, for they automatically obviated the possibility that factor W in its entirety is a purine or pyrimidine derivative. They likewise obviated the possibility that factor W is one or both of the pyridine nucleotides. Presumably the entire molecules of coenzyme and cozymase would not exist as such in liver extract unless the livers used for the preparation of the liver extract were heat-treated immediately after removal from the animal to destroy the enzymes present. The possibility that the substance or substances in the mercury filtrate constitute degradation products of the pyridine nucleotides is a very likely one, and a study of this possibility was our next step.

Attempts to isolate nicotinic acid amide, the prosthetic group of the pyridine nucleotides, from either the mercury precipitate or filtrate fractions have thus far been unsuccessful, but this may be due to the presence of interfering substances in our concentrates.

The possible growth-stimulating effect of crystalline nicotinic acid amide[2] has been under investigation for well over a year in this laboratory. The first levels tried were quite low because

[2] We are indebted to Dr. H. Adkins and Mr. E. L. Hutchinson for a supply of nicotinic acid amide.

nicotinic acid amide was thought to have toxic properties. Later when a growth response was noted with a combination of adenine nucleotide and nicotinic acid amide at quite low levels, we could not be certain whether the combination of the two was needed for growth or whether one substance alone was the active agent. An investigation of the substances singly and in combination at much higher levels was then carried out with very interesting results.

At the first level tried of only 50 micrograms per day there was no measurable response with nicotinic acid amide. Adenine sulfate and nicotinic acid amide together at 50 microgram levels proved inactive. When adenine nucleotide,[3] 200 micrograms, and

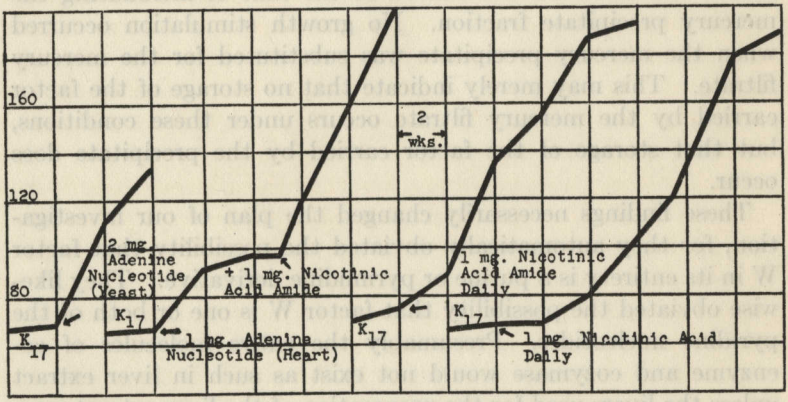

CHART VI. Growth curves for rats on basal Ration K_{17} plus adenine nucleotide, adenine nucleotide supplemented after growth had slowed with nicotinic acid amide, nicotinic acid amide alone, and nicotinic acid alone.

nicotinic acid amide, 80 micrograms, were fed daily, a slight stimulation ensued. Adenine nucleotide and nicotinic acid amide were then investigated singly and in combination at higher levels (Chart VI). Each substance produced a definite and measurable response, but the nature of the responses was quite dissimilar. Adenine nucleotide at 1 mg. daily supported slight growth for a few weeks. The growth response was immediate but not con-

[3] The adenine nucleotides used in this work were supplied to us by Dr. Mary V. Buell, the Johns Hopkins University. Two different nucleotides, heart nucleotide and yeast nucleotide, were tried. No great difference in activity has been observed thus far.

tinued. Nicotinic acid amide, as compared to this, at a 1 mg. level daily produced no significant increase during the 1st week or 2 but a rising and constant increase thereafter. Combination of nicotinic acid amide and adenine nucleotide, each at 1 mg. per day, allowed a more immediate and even response and the amount of increase was somewhat greater than in the case of the nicotinic acid amide alone. Nicotinic acid alone at a 1 mg. level resulted in a very delayed response and growth was not as great at any time as that obtained with the amide.

DISCUSSION

We feel that ample evidence has been presented in this paper to establish the existence of an essential factor or factors distinct from vitamin B_1, flavin, antipellagra factor, vitamin B_4, and vitamin B_6. The results also indicate that the metabolism of the additional factor is closely related to flavin. This would suggest that we might be dealing with coenzyme and cozymase. The isolation work, however, indicates that the concentrates do not contain the intact coferment. The preliminary results show that certain degradation products have growth-stimulating properties. The implications of these data are very interesting in view of the recent recognition of the importance of nicotinic acid amide and the pyridine nucleotides in biological processes (7).

There are certain reports in the literature dealing with the relation of nicotinic acid amide as well as the free acid to the growth of rats and carbohydrate metabolism but the results are not entirely in agreement.

In 1935 von Euler and Malmberg (8) using a ration similar to the Sherman-Borquin diet but supplemented with a vitamin B_1 concentrate and 10 micrograms of flavin per day per rat showed the complete inefficacy of highly purified preparations of cozymase and coenzyme in amounts of 1 mg. per day per rat. They obtained no growth with nicotinic acid or nicotinic acid amide at 1 mg. daily levels, using the same ration supplemented with vitamin B_1 and flavin. Neither did they obtain a growth response with adenylic acid or a high purine concentrate (Liebig's meat extract). The fullers' earth filtrate from a boiled yeast extract likewise proved inactive. The workers did find some indication that nicotinic acid amide might have some beneficial effect, since it prolonged the life of rats on deficient diets.

Funk (9) has reported that nicotinic acid and nicotinic acid amide administered orally or by injection had growth-promoting properties and food-sparing action for rats. Since the details of this work have not been published, it is impossible to compare these results with those we have obtained.

Very recently the essential nature of cozymase or coenzyme in the nutrition of *Hæmophilus parainfluenzæ* was demonstrated by Lwoff and Lwoff (10). This particular pathogenic type of bacterium cannot utilize nicotinic acid amide in place of cozymase as a growth stimulant. Knight (11) working with *Staphylococcus aureus* found nicotinic acid amide, nicotinic acid, and the ethyl ester of nicotinic acid all to be active growth factors. Since the amide was almost immediately active, the activity of the acid somewhat delayed, and the activity of the ester most delayed, a chemical change of the two latter forms to the amide was assumed to have occurred *in vivo*. Cozymase proved extremely potent in minute amounts and the growth response was immediate.

The delayed response to nicotinic acid amide and nicotinic acid which we have found to occur uniformly in rats, we believe, indicates that neither of these is the active growth principle, but that they must first be conjugated with something else in the body. Also the more delayed response elicited by feeding of nicotinic acid than that shown by the amide leads to the natural conclusion that the acid is first aminated *in vivo*. The apparently important part played by adenine nucleotide fits well into the theory that the pyridine nucleotides are the ultimate active growth principles and that rats are capable of synthesizing them from these constituent parts.

The separation of two supplementary fractions from factor W concentrates with mercury occurred almost coincidentally with our finding that adenine nucleotide had a supplementary growth effect with nicotinic acid amide. If present attempts to isolate adenine or its nucleotide from the precipitate and nicotinic acid amide from the filtrate prove successful, the very apparent similarity between growth responses obtained with pure adenine nucleotide and nicotinic acid amide and responses obtained with the mercury fractions from factor W preparations will be borne out.

Certain tests have been applied to all of our preparations in the hope that we might find some chemical characteristic intimately

connected with the biological activity of the effective moiety. As previously stated, all active preparations have strong reducing properties as measured by capacity to reduce Fehling's solution. Since many substances which could reduce alkaline copper might feasibly occur in all of our concentrates, this finding offers little basis for correlation. A strong pentose reaction is also obtained by Bial's and Tollens' tests with all active preparations and in many cases the amount of pentose has been quantitatively estimated according to the "Methods of analysis of the Association of Official Agricultural Chemists." Calculating the amount of pentose as ribose, we have found the mercury filtrate to contain about 4 times that of the mercury precipitate on equivalent samples. Since other furfural-producing substances than pentoses may be present in our preparations, this finding again does not offer conclusive evidence of the constituency of the active groupings.

Attempts to apply the Lane-Eynon and Shaffer-Hartmann titration methods for reducing sugars proved unwieldy and yielded variable results. A still further titration method for aldose sugars was then tried according to the method of Myrback (12) who had shown in 1935 that there was correlation between the lability to alkali and acid treatment of the reducing group of cozymase and its fermentation activity. Myrback had found that the Willstätter-Schudel alkaline iodine method gave a figure for glucose more than twice that given by the Shaffer-Hartmann method, but that this high reducing activity to iodine was more than 60 per cent destroyed by short heating with weak alkali and acid. Karrer *et al.* (13) in 1936 indicated the great capacity of synthetic pyridine bases to take up iodine and to lose this capacity by acid or alkaline heating. The latter reaction is thought to be quite specific for pyridine bases and its application to our preparations was made with this in mind. The mercury precipitate fraction first investigated gave reactions much like those reported by Myrback for impure preparations of cozymase. There was 29 per cent destruction of the reducing activity to alkaline iodine by 10 minutes heating at a pH of 10+. The calculation was made as glucose. The mercury filtrate fraction gave almost identical results, the destruction as glucose amounting to 30 per cent, although in this case the heating was continued for ½ hour.

The significance of the strong reducing properties of factor W

preparations is not known, but would appear to be related to both the content of pentoses and the presence of pyridine bases.

SUMMARY

1. Further evidence is presented for the existence of a water-soluble vitamin in liver preparations which is distinct from the known vitamins. This factor has been designated temporarily factor W.

2. Normal growth is obtained in rats fed the basal Ration K_{12} only when adequate amounts of both flavin and factor W are supplied.

3. A method for the preparation of a concentrate of factor W from the 92 per cent alcohol filtrate from liver extract is described.

4. The activity of the factor W concentrates seems to be destroyed when attempts are made to adsorb the factor on charcoal. It is very stable to acid and alkali in the cold, and complete destruction does not take place even by boiling for short periods at pH 1 and pH 9.0.

5. When attempts were made to precipitate the active substances with mercury according to the method of Warburg, both the precipitate and filtrate gave growth responses but optimum growth was obtained only when both fractions were fed. Further indication of the multiple nature of factor W was obtained from work with barium fractions.

6. Definite growth responses have been obtained by the addition of adenine nucleotides and nicotinic acid amide to the basal Ration K_{12} supplemented with flavin. Adenine nucleotides alone produced an immediate but not continuous response. Nicotinic acid amide alone gave a very poor response at first but after about 2 weeks a very definite and prolonged increase in the rate of growth resulted. The combination of the two supplements gave a more immediate and continuous response.

7. The relation of these results to the recent work on the structure of cozymase and coenzyme is discussed.

BIBLIOGRAPHY

1. Elvehjem, C. A., Koehn, C. J., Jr., and Oleson, J. J., *J. Biol. Chem.*, **115**, 707 (1936).
2. Elvehjem, C. A., and Koehn, C. J., Jr., *J. Biol. Chem.*, **108**, 709 (1935).
3. Halliday, N., and Evans, H. M., *J. Nutrition*, **14**, 45 (1937).

4. Birch, T. W., György, P., and Harris, L. J., *Biochem. J.*, **29**, 2830 (1935).
5. Day, P. L., Darby, W. J., and Langston, W. C., *J. Nutrition*, **13**, 389 (1937).
6. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
7. See Lipmann, F. (p. 19), Linderstrøm-Lang, K. (p. 43), and Chrometzka, F. (p. 211), in Luck, J. M., Annual review of biochemistry, Stanford University, **6** (1937).
8. von Euler, H., and Malmberg, M., *Biochem. Z.*, **283**, 455 (1936).
9. Funk, C., and Funk, I. C., *Proc. Am. Soc. Biol. Chem.*, **8**, xxxv (1937) (*J. Biol. Chem.*, **119** (1937)).
10. Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London, Series B*, **122**, 352 (1937).
11. Knight, B. C. J. G., *Biochem. J.*, **31**, 371 (1937).
12. Myrback, K., *Z. physiol. Chem.*, **233**, 95 (1935).
13. Karrer, P., Schlenk, F., and von Euler, H., *Ark. Kemi, Mineral. o. Geol.*, **12 B**, No. 26 (1936).

4. Birch, T. W., György, P., and Harris, L. J., *Biochem. J.*, **29**, 2830 (1935).
5. Day, P. L., Darby, W. J., and Langston, W. C., *J. Nutrition*, **13**, 389 (1937).
6. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
7. See Lipmann, F. (p. 19), Linderstrøm-Lang, K. (p. 43), and Chrometzka, F. (p. 211), in Luck, J. M., Annual review of biochemistry, Stanford University, **6** (1937).
8. von Euler, H., and Malmberg, M., *Biochem. Z.*, **283**, 455 (1936).
9. Funk, C., and Funk, I. C., *Proc. Am. Soc. Biol. Chem.*, **8**, xxxv (1937) (*J. Biol. Chem.*, **119** (1937)).
10. Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London, Series B*, **122**, 352 (1937).
11. Knight, B. C. J. G., *Biochem. J.*, **31**, 371 (1937).
12. Myrback, K., *Z. physiol. Chem.*, **233**, 95 (1935).
13. Karrer, P., Schlenk, F., and von Euler, H., *Ark. Kemi, Mineral. o. Geol.*, **12 B**, No. 26 (1936).

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