Studies on Vitamin B₂ Complex.

III—Further Studies on the Growth-promoting Factor in Liver Extract.

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In the previous paper,⁽¹⁾ dealing with the vitamin B₂ complex, I presented the evidence for the existence of growth-promoting factor (or factors) which could be separated from liver extract by treating its concentrated aqueous solution, after an exhaustive adsorption with acid clay, with large volumes of a mixture of alcohol and ether. This factor may be similar to or identical with factor W described by Frost and Elvehjem.⁽²⁾

In this paper I wish to present further proof that the profound growth-stimulating property of the alcohol-ether insoluble fraction is due to a factor distinct from riboflavin and to give some properties.

EXPERIMENTAL.

The basal ration described previously was slightly modified, and it has the following composition, supplemented daily with 1 drop of cod liver oil:

Purified fish protein	20%
Dextrinized corn starch	53
White corn	15
Butter fat	8
McCollum's salt	4

From the statement of Frost and Elvehjem,⁽²⁾ we can assume that this ration supplies sufficient amounts of vitamins B₁ and B₆, but is low in flavin and factor W. If flavin were only deficiency in this ration, normal growth should result when flavin is added. No such result appeared as seen in Fig. 1 (b). On the other hand, the striking effect on the growth was obtained by supplementing the liver concentrate to flavin (Fig. 1 (c) and (d)). If the liver growth factor was alone added, there was good growth for some interval, but followed thereafter by decline. At this condition, if flavin was added, there was immediate resumption of growth. If both flavin and liver growth factor were added from the beginning to the diet, there was profound growth response. These results demonstrated very clearly that there is a remarkable supplementary action between flavin and liver growth factor in the nutrition of rats (Fig. 2 (b), (c) and (d)).

METHODS AND RESULTS.

Young rats weighing between 40 and 50 g were placed on the basal diet and each received daily 20 γ of vitamin B₁ hydrochloride. After being constant in

weight for several days, the rats received the test doses of the different fractions supplemented by 20 γ of flavin daily. The control animals received flavin only. Three to six rats were used for each test and growth was observed for a period of six weeks. Some variations had been noted occasionally in the growth responses of rats from different litters, but within a litter, growth responses were fairly regular.

Flavin was prepared from dried egg-white powder extracting with a mixture of methanol-water (3 : 1), and concentrating the extracts under diminished pressure. The amounts of flavin in the concentrate thus obtained was estimated as lumiflavin by Pulfrich's photometer. This raw flavin was provided for rats at the level corresponding to 20 γ of pure flavin.

Preparation of the growth factor from liver extracts.

(a) Koehn and Elvehjem,⁽³⁾ Macrae and Edgar⁽⁴⁾ have reported that growth promoting factor for rats can be extracted from liver concentrate with amyl alcohol. The following procedure, therefore, was carried out: 300 cc of liver extract,⁽¹⁾ free from flavin (1 cc corresponding to 3 g of dry liver) previously treated with acid clay at pH 3.0, was adjusted to pH 1.4 with H_2SO_4 and extracted five times with 500 cc of amyl alcohol. The amyl alcohol solution was shaken three times with 400 cc of 10% NaOH. The alkali extracts were combined and, after neutralizing with HCl, the amyl alcohol was completely removed in vacuo. The effect, however, of the amyl alcohol extract on animal growth, as seen in Fig. 2(a) and (a'), was not so good as that with acidified aqueous acetone extract obtained by the method described in the following: Two litres of the liver concentrate* (1 cc equivalent to 2 g of fresh beef liver), previously extracted with a mixture of methanol-water, acidified with H_2SO_4 , and then treated with acid clay to remove flavin, having an acid reaction of a pale green-yellowish colour was treated twice with 100 g of acid clay each time to remove a trace of flavin which might remain in the solution, then the filtrate from acid clay adsorbates was neutralized with NaOH, and concentrated to about 200 cc in vacuo. Into the dark thick solution, 92% ethyl alcohol was added and the resulting precipitates were separated from the clear alcoholic solution. After similar treatment was repeated twice more, the combined alcoholic solution was evaporated. The 92% alcohol-soluble liver fraction was now placed in 300 cc of 0.2% HCl aq. solution and 2700 cc of acetone was added with constant stirring. The acetone fraction was decanted off and the residue was treated in the same way with a mixture of 600 cc of 0.2% HCl aq. solution and 900 cc of acetone. The similar procedure was repeated five times more. The acetone was recovered from the combined extracts by distillation. The resulting dark, aqueous solution was diluted with distilled water, considerable denatured protein separated out. This was filtered and the clear brown solution free from flavin was concentrated to about 200 cc for use and stored in the refrigerator.

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*Some experiments of the chemical properties of
the growth-promoting factor.*

1. Adsorption on charcoal — As I found that the acidified aqueous acetone filtrate contained the active principle, charcoal adsorption according to the method of Edgar et al.⁽⁵⁾ was carried out: The solution was adjusted to pH 2.5 with HCl and treated with charcoal. The charcoal was washed with 0.1 *N* HCl and then eluted with 0.25 *N* NaOH, being allowed to stand overnight in the ice box. The elution was repeated once more. The eluate and filtrate were fed by separatory or combination at high levels; neither of them showed any appreciable growth-stimulating effect (Fig. 3 (a)).

2. Precipitation with acetone — The 92% alcohol-soluble fraction diluted with water of half volume of the solution was mixed with acetone. After standing overnight in the cold, the acetone solution was decanted from the gummy precipitates. After repeating similar treatment once more, the combined acetone was removed in vacuo. The greater part of activity was found in the soluble part, whilst the precipitates appeared inactive (Fig. 3 (b)).

3. Phosphotungstic acid precipitation — 60 cc of the 92% alcohol-soluble fraction diluted with water, acidified with H₂SO₄ to pH 1, and a 20% phosphotungstic acid solution was added until there was no further precipitate. After standing 24 hours with occasional stirring, the precipitates were filtered off. The filtrate, after removal of the excess phosphotungstic acid by the usual means, was found to have vitamin activity approximately equal to that of the starting alcohol-soluble fraction. The phosphotungstic precipitate was decomposed by H₂SO₄ and its filtrate was also tested, showing negative results (Fig. 3 (c)).

4. Barium precipitation — 100 cc of the acetone filtrate were diluted with an equal volume of distilled water and treated with a solution of 10 g of barium

Fig. 1. Growth curves for rats on basal ration (a), basal ration + flavin (b), basal ration + LE (c) and basal ration + LEA (d). LE and LEA are liver concentrate, free from flavin, and 92% alcohol-soluble liver extract, respectively. (1:20) and (1:40), show that 1 part each of the preparation corresponds to 20 and 40 parts of fresh liver,

acetate in 25 cc of H₂O and the formed gummy precipitates were removed by filtering. The filtrate made alkaline to phenolphthalein with a cold, saturated barium hydroxide solution, and the slight precipitate was removed as before. The filtrate was then mixed with a 3-fold volume of alcohol and the formed flocculous precipitate centrifuged out. This precipitate was immediately dissolved in 50 cc of H₂O. After removal of barium quantitatively with sulphuric acid, the concentrate was stored in the ice-box. The alcoholic filtrate was

also freed of barium with sulphuric acid and prepared for assay. The feeding results of these fractions singly and together were given in Fig. 3 (d).

Fig. 2. Growth curves of rats receiving flavin alone, liver concentrate free from flavin, the combination of the two, and flavin + amyl alcohol extract of liver = A* (1 cc of A* is equivalent to 3 g. of dry liver).

Fig. 3. Growth curves showing the effect on rats receiving various fractions from liver extract.

Can nicotinic acid replace the liver growth factor?

Recently the importance of nicotinic acid and its amide in the biological process is recognized, and there is some evidence that the acid or its amide has a growth-promoting action for rats or pigeons on various diets deficient in some factors of the vitamin B complex, while the others claim that these pyridine compounds have no significant effect on the growth of rats receiving vitamin B₁ and flavin as source of vitamin B complex. It is interesting, therefore, to find if nicotinic acid can replace the growth factor in liver extract. The experiment was carried out as follows: The rats received the basal diet supplemented with vitamin B₁. When they ceased to grow, the rats were divided into two groups. One group of the rats received 20 γ of

Fig. 4. Growth curves showing the inability of nicotinic acid. At point A, LEA is substituted for nicotinic acid.

the rats were divided into two groups. One group of the rats received 20 γ of

flavin and 2 mg. of nicotinic acid daily, while the other group was given, in addition to 20 γ of flavin daily, 2% of the 92% alcohol-soluble liver extract. The results in Fig. 4 showed that nicotinic acid had no significant increase in the growth rate and the acid could not be substituted for the growth factor found in the liver extract.

DISCUSSION.

The observations described above confirm the previous finding⁽¹⁾ that in addition to vitamins B₁, B₄(?), B₆ and flavin, the rat requires still another factor (factor W?) to attain the maximum growth. This factor can be prepared from liver concentrate by dissolving in 92 per cent ethyl alcohol and treating the alcohol-soluble fraction with acidified aqueous acetone. The active factor is not adsorbed by acid earth at pH 1.4 and 3.0 or by charcoal at pH 2.5. The factor is readily soluble in ethyl alcohol and partially precipitated by barium salt, but not by phosphotungstic acid. Recently Edgar and Macrae⁽⁶⁾⁽⁸⁾ described that the yeast-extract filtrate factor for rat is probably acidic, since it is precipitated by barium hydroxide in 90 per cent alcohol. In a recent paper Frost and Elvehjem⁽²⁾ reported evidence suggesting a relation of factor W to pyridine nucleotides.

The comparison of the properties of the fraction, as already stated, in an aqueous acetone solution indicates that the factor promoting growth in rats is probably identical with factor W described by Frost and Elvehjem.⁽²⁾

The biological importance of the pyridine compounds, was recently recognized by the demonstration of Warburg and Eular that nicotinamide is the active group in certain coenzymes. Funk and Funk,⁽⁷⁾ Frost and Elvehjem⁽²⁾ showed that nicotinamide, and nicotinic acid and adenine nucleotides have growth-promoting action for pigeons and rats on certain rations. I have found, however, that nicotinic acid has no stimulating effect on the growth of rats, in agreement with the results of Edgar and Macrae,⁽⁴⁾ who showed that the pyridine compounds cannot replace their yeast-filtrate factor or yeast-eluate factor, although they have isolated nicotinamide from the yeast-eluate fraction. Moreover, these workers demonstrated the presence of a growth factor for rats distinct from nicotinic acid in an amyl alcohol extract of liver and described that this factor may be identical with the yeast-filtrate factor. Dann and Subbaraw⁽⁸⁾ reported that nicotinic acid does not cure rat dermatitis or prevent chick dermatitis and concluded that vitamin B₆ and the filtrate factor are both distinct from nicotinic acid. Recently Elvehjem and his co-workers claimed that a factor was isolated from liver and identified as nicotinic acid amide.⁽⁹⁾ More recently Elvehjem and his colleagues⁽¹⁰⁾ reported that nicotinic acid or its amide has no curative power on chick dermatitis and that liver extract contains a factor necessary for the prevention of a typical dermatitis in chick which is separate and distinct from vitamin B₁, vitamin B₆, flavin, nicotinic acid, and factor W.

Regarding the nature of the growth factor (factor W?), I wish to cite the claim of Frost and Elvehjem⁽²⁾ on the relationship between factor W and pyridine nu-

cleotides and on the chemical behaviour of the factor towards mercury and barium salts which indicated a similarity to the coenzymes. We must consider, however, the comment of Peters⁽¹¹⁾ who stated: "at present results on factor W are difficult to reconcile with other finding owing to the non-synthetic nature of the diet. Vitamins B₁ and B₆ were provided in the form of white corn composing 12 per cent of the diet which, it is stated by Frost and Elvehjem, also contain small yet adequate amount of the filtrate factor". Further experiments are, therefore, needed with regard to the nature of the growth-stimulating factor in liver.

SUMMARY.

1. A method for separation and concentration from liver extract of the growth-promoting factor (or factors) distinct from flavin is given.

2. The factor (or factors) is readily soluble in ethyl alcohol, and the addition of a large volume of acetone to the aqueous solution cannot precipitate the active factor. The factor is not precipitated by phosphotungstic acid, but partially by barium. It is not adsorbed by acid clay or charcoal at pH 1.4 and 2.5.

3. Normal growth is induced in rats when both flavin and liver growth factor are added to the basal ration.

4. Nicotinic acid cannot replace the active factor, as tested on growth of rats.

5. Comparison of the properties of the factor indicates that it is distinct from riboflavin, but may perhaps be identical with factor W described by Frost and Elvehjem.

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