ISOLATION OF THE UNIDENTIFIED GROWTH FACTOR
(VITAMIN B_{12}) IN DISTILLERS' DRIED SOLUBLES*

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Recent investigations have shown that distillers' dried solubles contain
a factor or factors which are necessary for the growth of chicks (1) and
rats (2). This factor is distinct from vitamin A, vitamin D, thiamine,
riboflavin, inositol, nicotinic acid, pantothenic acid, p-aminobenzoic acid,
choline, pyridoxine, biotin, folic acid, or 2-methylnaphthoquinone.

A procedure has been developed for the isolation of this unidentified
growth factor in a highly concentrated condition. This method is based
upon physical and chemical characteristics of the active principles which
were discovered during the fractionation studies (2).

EXPERIMENTAL

A method was developed for the isolation of the unidentified growth
factor in distillers' dried solubles, which involved the following essential
procedures: extraction of distillers' dried solubles with acidified water, re-
moval of proteins, differential chromatographic adsorption of impurities,
precipitation, and the separation of the active principle with an immiscible
solvent. The activity of the various fractions was determined by the rat
growth method previously described (2).

Distillers' dried solubles were extracted with 3 liters of 0.1 \text{n} HCl per
kilo of solubles by autoclaving for 30 minutes at 120°. This preparation
was cooled, adjusted to pH 6.5, and filtered. The residue was reextracted
twice, after which it was washed with distilled water by stirring and again
filtered. The combined filtrates were concentrated under reduced pressure
to the equivalent of approximately 200 ml. per kilo of original material and
adjusted to pH 3.5.

To precipitate protein and other inert material, this concentrate was
poured into 3 volumes of ethanol, stirred for several minutes, allowed to
stand overnight, and filtered. Under these conditions, factor S (3) should

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ISOLATION OF UNIDENTIFIED GROWTH FACTOR

have been removed. The filtrate was adjusted to pH 7, stirred, allowed to stand overnight, and again filtered. This procedure is reported to remove factor R (3).

The filtrate was slightly acidified with hydrochloric acid, concentrated under reduced pressure to about 150 ml. per kilo of original material, and adjusted to pH 6.5. This solution was filtered through a column (4 cm. X 10 cm.) of fuller's earth1 diluted with Celite (1:1) which removed some impurities without adsorbing the active principle (2).

The filtrate was concentrated under diminished pressure to about 100 ml. per kilo of the original solubles. After adjusting the solution to a hydrochloric acid content of 3.5 per cent, sufficient solution of phosphotungstic acid (30 gm. per 100 ml. of 3.5 per cent HCl) was added for complete precipitation. The contents of the flask were heated to dissolve most of the precipitate, cooled, and placed in the refrigerator for 48 hours. The cold solution was filtered through a cold Büchner funnel with a hardened filter paper (Whatman No. 50). The precipitate was washed several times with a cold solution containing 2.5 per cent phosphotungstic acid, and 3.5 per cent hydrochloric acid. The filtrate was found to be biologically inactive, and was discarded.

The precipitate was dissolved in a slightly alkaline solution of sodium hydroxide, and the phosphotungstic acid precipitated with barium chloride. The barium phosphotungstate was removed by filtration, and washed with hot water.

After concentration, the aqueous solution was extracted repeatedly with chloroform in a separatory funnel. The combined chloroform extracts were concentrated to dryness under reduced pressure. A yellow-orange non-crystalline residue remained. This material was found to be readily soluble in water, acetone, ethanol, ethyl ether, chloroform, and benzene. When this material was dissolved in water at a concentration of 100 γ per ml., it appeared practically colorless and showed fluorescence under an ultraviolet lamp.

To determine its biological activity, this material was added to the basal ration at approximately 250 γ per 100 gm. of ration and tested with rats as previously described. A good growth response was obtained (Table I).

Since the aqueous solution was almost colorless, its spectral absorption characteristics were studied. A Beckman spectrophotometer, equipped with a hydrogen arc and quartz cells, was used at wave-lengths between 2400 and 4000 A. To determine whether the absorption curve bore any relation to the active component from distillers' solubles, concentrates were prepared from other active materials and their spectral characteristics were compared (Fig. 1.). Concentrates prepared from rice polishings concentrate

1 City Chemical Corporation, New York.
TABLE I

<table>
<thead>
<tr>
<th>Concentrates added to basal ration*</th>
<th>Average gain per wk.† (8 rats per lot)</th>
<th>g/m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>17.2</td>
</tr>
<tr>
<td>Distillers' solubles (Seagram)</td>
<td></td>
<td>29.8</td>
</tr>
<tr>
<td>Liver extract (Lilly)</td>
<td></td>
<td>27.8</td>
</tr>
<tr>
<td>Rice polishings (Labco)</td>
<td></td>
<td>28.0</td>
</tr>
</tbody>
</table>

* In amounts equivalent to approximately 10 per cent of the original material.
† Least significant difference, 5 per cent level 2.5 g/m., 1 per cent level 3.4 g/m.

![Absorption Spectra](image)

**Fig. 1.** A comparison of the absorption spectra of the isolated concentrates from distillers' dried solubles, rice polishings concentrate (Labco), and liver extract (Lilly).

(Labco) and liver extract (Lilly) were diluted so that at the maximum of 2820 Å they gave densities slightly less than that obtained with the preparation from the distillers' solubles. The biological activities of these con-
centrates (Table I) established a parallelism between the characteristic curves and activity.

After it was found that maximum growth response was produced with 250 γ of the isolated material from solubles per 100 gm. of the ration, a biological assay was performed to determine the minimum quantity necessary for a definite growth response and the amount required for a maximum biological response.

The biological assay was performed with 50 rats, equally divided according to sex. Instead of incorporating the factor into the ration, it was fed as a daily supplement. In a preliminary assay, it was found that levels of 10, 20, 30, and 40 γ per day were equally effective in stimulating growth. This indicated that the effective dose was below 10 γ per day. Therefore levels of 0, 2, 4, 6, and 10 γ per day were fed. Ten rats were fed at each level. The growth response curve is given in Fig. 2. The maximum biological effect was obtained with 10 γ of the factor per day. Definite growth stimulation was obtained with 2 γ per day.

Since the characteristic absorption spectra of the concentrates from the three different sources were practically identical, exhibiting a maximum at 2820 Å, and inasmuch as they were biologically active, it is apparent that the isolation of the unidentified growth factor in a high state of purity has been accomplished.

**DISCUSSION**

Although previous experiments (1, 2) gave strong evidence for the existence of an unidentified growth factor in distillers' dried solubles which was
distinct from the known vitamins and postulated factors, these experiments offer proof that the observed growth responses were due to a definite substance which has been isolated in a state of relatively high purity. This is substantiated by its characteristic absorption spectrum and by its high biological activity. Since the potency of this product is comparable to that of a number of vitamins, it appears logical to class this factor with the other vitamins of the B complex. It is tentatively called vitamin B13.

A number of properties are now known. It is stable to heat, acid, and alkali. It is soluble in water, acetone, chloroform, ethanol, ethyl ether, and benzene. It is precipitated by phosphotungstic acid and lead acetate. It is not adsorbed on fullers' earth or Darco but is adsorbed from acid solution on Florisil, Lloyd's reagent, norit, and Decalso. Its absorption spectrum exhibits a maximum at 2820 A.

The solubility of this factor in chloroform as well as ether adds further evidence that it is not the cow manure factor (4). It is possible that it may be similar to one of the fractions obtained from liver extract by Barton-Wright et al. (5). They obtained a fraction by the extraction of an aqueous solution with chloroform, which stimulated growth of Lactobacillus helveticus and Streptococcus lactis. No tests were reported with animals.

SUMMARY

A new growth factor for rats, tentatively called vitamin B13, has been obtained from distillers' dried solubles, rice polishings concentrate, and liver extract in a non-crystalline but highly purified state. 2 γ of this substance give definite growth stimulation and 10 γ per day give the maximum effect.

Spectral absorption curves showing a maximum at 2820 A were obtained with concentrates of high potency from each of the three materials, distillers' dried solubles (Seagram), rice polishings concentrate (Labco), and liver extract (Lilly).

The procedure for isolation involves extraction with 0.1 N hydrochloric acid, precipitation of protein and other inert material with ethanol, chromatographic adsorption of impurities on fullers' earth, precipitation of the active factor with phosphotungstic acid, and separation by chloroform extraction.

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