IDENTIFICATION OF THE MOUSE ANTIALOPECIA FACTOR

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(Received for publication, January 11, 1941)

Recently a new dietary essential required by the mouse has been described (1). When this substance was absent from the diet, young mice soon ceased to grow and became completely bald over large areas of the body. Preliminary concentration of the curative substance which was present in liver has been described. Norris and Hauschildt (2) simultaneously and independently described a similar syndrome in mice which were fed a highly purified diet. In the present communication the identification of the curative material will be described. A preliminary statement of our results has appeared recently (3).

EXPERIMENTAL

Assay Technique—In the early part of this investigation the assay procedure previously described (1) was followed exactly. In the last stages, especially after the crystallization of the active substance, the following modifications were introduced. First, the administration of yeast extract to the depleted animals (as with Diet Y) was discontinued and a purely synthetic mixture of water-soluble vitamins (as in Diet S) was employed throughout the test period. In addition, some of the latest experiments were done with 5 mg. of dl-sodium pantothenate per 100 gm. of ration in place of the 1 mg. level used previously. The quantity of pantothenic acid had a decided influence on the occurrence and course of alopecia and, when sufficiently high levels were fed, the disease frequently did not result even in the absence of the present antialopecia factor. However, since the relationship between pantothenic acid and the antialopecia factor requires more study,
the details of our investigation of this relationship will be communicated subsequently.

The omission of yeast extract had one noteworthy result. The restoration of hair did not occur as rapidly in animals on a purely synthetic diet as in those fed the yeast extract ration. Yeast extract could not be included from the beginning of the experiments, for, when this was done, no alopecia resulted. For example, two groups of six mice each were fed the purified ration plus 2 per cent of yeast extract from the beginning of the experiment and no cases of alopecia were observed. This fact suggested that the yeast extract was not devoid of the antialopecia factor.

Preliminary Concentration—A statement of the steps employed in bringing about concentration of the active substance will be made in order to illustrate the course of the reasoning which led to the use of phytin. Actual details of the isolation of the active principle will be described below after the effect of phytin has been noted. The source of the vitamin in every case has been the fraction of aqueous liver extract which was insoluble in 70 per cent alcohol; this was the same fraction as previously used. It was designated Fraction A.\(^1\) This material was dissolved in water, dialyzed, and the non-dialyzable portion treated with norit. The active norit filtrate was made alkaline with barium hydroxide and alcohol was added in order to precipitate the active compound. The precipitate was freed of barium and the active substance was rendered dialyzable by heating it with sodium hydroxide. Concentrates prepared in this manner gave the Scherer test for inositol.

Effect of Phytin—The properties of the concentrates suggested that the active substance might possibly be some phosphoric acid ester of inositol. Studies on the distribution of the vitamin in natural products had revealed that cereal grains were relatively rich sources. Thus, for example, 2 per cent of oats was sufficient to bring about slow cure of alopecia. For these reasons it was thought justifiable to test the action of phytin. It was thought at the time that the active substance was a lower ester of inositol but that phytin might possess activity. When 100 mg. of phytin per 100 gm. of ration were fed, hair was restored and resumption of growth occurred.

\(^1\) We wish to thank Dr. David Klein of The Wilson Laboratories for gifts of this material.
Isolation of Antialopecia Factor—While the assays of phytin were in progress, a crystalline material was obtained from our best concentrate by precipitation with lead acetate and ammonia followed by purification with norit and crystallization from alcohol. The crystals melted at 214–216° and contained 39.8 per cent carbon. When fed at a level of 100 mg. per 100 gm. of ration, they caused restoration of hair. Subsequent tests with authentic inositol showed that this material also possessed activity.

Many procedures have been tested for the isolation of inositol from liver Fraction A and the one found most satisfactory will be described. 100 gm. of Fraction A were dissolved in water and dialyzed for 18 hours. The non-dialyzable portion was evaporated to 200 cc. and refluxed with 400 cc. of concentrated HCl for 6 hours. The solution was then concentrated under reduced pressure to a syrup, made alkaline with barium hydroxide, and treated with sufficient alcohol to give a final concentration of 75 per cent. The precipitate was filtered off, washed with alcohol, and decomposed by suspending it in 65 per cent alcohol and passing in carbon dioxide. The filtrate from the barium carbonate was concentrated under reduced pressure to about 200 cc. and treated with saturated lead acetate until no more precipitate formed. The precipitate was removed and the resulting filtrate was treated with 100 cc. of saturated lead acetate. Enough ammonia was added to cause complete precipitation. The precipitate was filtered off and washed and then decomposed with a slight excess of sulfuric acid. Lead sulfate was removed and the resulting filtrate was again made alkaline with barium hydroxide dissolved in methyl alcohol and enough ethanol was added to give a final concentration of alcohols of 70 per cent. After the mixture had stood overnight, the precipitate was filtered off, washed, and then decomposed by suspending it in 70 per cent alcohol and passing in carbon dioxide. The barium carbonate was filtered off; the filtrate was concentrated to a small volume under reduced pressure, acidified with sulfuric acid, and filtered through norit. Alcohol was added to the filtrate until crystallization occurred. The crystals were recrystallized from water by the addition of alcohol. 42 mg. of material were obtained which melted at 218°.

Inositol in the same bath melted at 218°.

C₆H₁₂O₆. Calculated, C 40.0, H 6.7; found, C 40.2, H 6.7
10 mg. of the crystals were heated with 20 mg. of sodium acetate and 20 cc. of acetic anhydride. The excess anhydride was removed under reduced pressure, the sodium acetate was removed by extraction with water, and the product was recrystallized twice from dilute pyridine; m.p. 212-213°. Inositol hexaacetate in the same bath melted at 213°.

\[ \text{C}_{13}\text{H}_{24}\text{O}_{12} \] Calculated, C 50.0, H 5.5; found, C 49.5, H 5.2

**Effect of Inositol**—Simultaneously with the assay of the inositol isolated from liver, authentic inositol was tested. 100 mg. per

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Level</th>
<th>No. of recoveries in 18 days</th>
<th>Average gain in weight during test period (gm. per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Phytin</td>
<td>100</td>
<td>1*</td>
<td>0.4</td>
</tr>
<tr>
<td>Crystals from liver</td>
<td>100</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Inositol</td>
<td>100</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Phosphorylated inositol</td>
<td>100</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Inositol purified through acetate</td>
<td>100</td>
<td>2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Recovery not apparent until after 4 weeks on phytin.

100 gm. of ration were sufficient to bring about restoration of hair and resumption of growth. The inositol (1 mole) was then phosphorylated by heating with pyridine and phosphorus oxychloride (7 moles) and, when this material was purified and assayed, it was found to be as active as the original inositol. Lower levels of inositol have been tried and on the yeast extract ration cures have been obtained with as little as 10 mg. per 100 gm. of ration (Table I).

Since inositol is isolated from natural sources and since relatively large amounts must be fed, it is difficult to prove that a small
amount of impurity is not the active material. In addition, slight change of activity with recrystallization cannot be detected, since no quantitative procedure for the assay of the antialopecia factor is available. It is only possible to observe qualitatively whether or not a substance is active. Recrystallization of inositol from dilute alcohol did not destroy its potency. Furthermore, careful purification of inositol hexaacetate by recrystallization from pyridine and then from alcohol, when followed by hydrolysis and further recrystallization of the free alcohol, did not destroy the activity. For these reasons it is believed that inositol or its phosphoric acid ester is the antialopecia factor.

Since only a small amount of inositol could be isolated from the liver fraction and since the amount obtained was not sufficient to account for the observed potency of the liver, a recovery experiment was performed. 100 mg. of inositol were added to the non-dialyzable portion of 100 gm. of Fraction A and the procedure described above was repeated. 56 mg. of inositol were obtained. It was thus evident that only a small fraction of the inositol present was isolated by our procedure.

DISCUSSION

The experiments related above demonstrate that the growth of hair of mice is markedly influenced by inositol or its esters. They further demonstrate that a combined form of inositol occurs in liver. Combined inositol, especially in heart muscle, has been postulated by Winter (4) and by Rosenberger (5) based on amounts isolated before and after autolysis or treatment with alkali. The present work demonstrates that an alcohol-insoluble, water-soluble, non-dialyzable substance occurs in liver which yields inositol upon acid or alkaline hydrolysis. That no free inositol was present in the non-dialyzable concentrates was shown by failure to isolate crystals when acid or alkali treatment was omitted from the procedure.

The relationship of inositol to the growth of hair in the mouse suggests the use of this substance in other species which manifest deficiencies involving the hair. Whether or not inositol is the additional anti-gray hair factor postulated by Dimick and Lepp (6) and by Williams (7) has not been determined.
SUMMARY

The alopecia which developed in young mice raised on a highly purified diet was cured by addition of inositol or of phytin to the ration. Inositol has been isolated and identified in liver concentrates which cure the same type of alopecia.

Observations on combined inositol in liver have been made.

BIBLIOGRAPHY

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J. Biol. Chem. 1941, 139:29-34.

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