SYNTHESIS OF INOSITOL IN MICE

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Since it has been shown (1, 2) that inositol possesses vitamin activity, it has been desirable to investigate the metabolism of this compound. Some studies have been made previously, but the work has been seriously limited by the lack of suitable methods of quantitative estimation. With the development of a satisfactory micromethod for the estimation of inositol (3), it has been possible to study the metabolism of this substance in some detail. In particular, it has been possible to show that while mice require inositol in the diet, they are able to synthesize the compound when none is ingested. Furthermore, the site of synthesis has been indicated, and a possible explanation for the numerous spontaneous cures of alopecia (4) has been discovered. Finally, the influence of pantothenic acid on the metabolism of inositol has been demonstrated.

EXPERIMENTAL

Method of Analysis.—Inositol determinations were made according to the method recently described by Woolley (3). Individual mice were killed with chloroform, weighed, and suspended in 50 cc. of HCl of such concentration that the final suspension was 18 per cent HCl. The mixture was refluxed for 6 hours and extracted twice with ether. The analysis for inositol was then conducted on the aqueous phase as previously described. In every case in which animals had received a diet containing inositol they were fed a ration free of this substance for 3 days before analysis. This was done in order to remove ingested inositol from the intestinal tract.

Variation in the values observed for individual male weanling mice is illustrated by the data given in Table I.

Synthesis of Inositol in Mice

In order to determine whether mice were able to synthesize inositol, the following experiments were performed.

Forty-five weanling male mice were fed a highly purified diet composed of sucrose, inorganic salts, casein, cod liver oil, corn oil, thiamin, riboflavin, vitamin B_6, nicotinic acid, choline, and pantothenic acid. The composition of this diet has been described (2).

Ten mice were individually analyzed and the average inositol content of a weanling mouse was thus obtained (see Table II). After the animals had been

fed the purified ration for 2 weeks ten more animals were analyzed. A similar group was analyzed at 4 weeks and another group of five at 6 weeks. The inositol content of an average mouse at biweekly intervals on this diet is shown in Table II. It can be seen that the mice increased in total content of inositol per mouse even though none of this substance was ingested. For purposes of comparison the average inositol content of six mice raised on stock rations for 4 weeks after weaning is included in Table II. The inositol content of six

TABLE I

Inositol Content of Individual Weanling Male Mice

Mouse No.	Live weight	Inositol content
	gm.	*gamma/mg.*
240	7.5	0.50
241	8.5	0.50
242	8.5	0.47
240A	8.5	0.47
345	7.5	0.48
340	7.5	0.50
341	8.5	0.50
342	8.5	0.47

TABLE II

Inositol Content of Mice Fed Rations with and without Inositol

Description of mice	Average live weight	Inositol content	Total amount of inositol
	gm.	*gamma/mg.*	*mg.*
Weanlings	7.0	0.49	3.4
2 wks. without inositol	15.0	0.35	5.3
4 wks. without inositol	24.0	0.36	8.6
6 wks. without inositol	35.0	0.35	12.3
4 wks. on stock ration	25.0	0.39	9.8
4 wks. with inositol	23.0	0.54	12.4

animals fed the highly purified ration plus 100 mg. of inositol per 100 gm. of ration for 4 weeks after weaning is also shown.

The experiment was repeated with a second group of nine mice. These animals increased in inositol content on the average from 4 mg. to 12 mg. in 6 weeks. The experiment was again repeated using eight animals. These increased in total inositol content from 4 mg. to 11.4 mg.

In the presence of adequate pantothenic acid one characteristic of inositol deficiency was the variation in gain of weight which individual animals exhibited. Another characteristic was that, at about the 6th week of the experimental period, many showed a precipitous loss of weight. Unless inositol was fed when this loss of weight began, death resulted. The rate of loss was

as great as the rate of gain had been before the decline began. A mouse typical in this respect was No. 313, which gained from 9.5 gm. to 29.5 gm. in 5 weeks. In the following 10 days the weight declined to 21.5 gm. 100 mg. of inositol per 100 gm. of ration were added, and in the next 8 days the mouse increased in weight to 27.0 gm. When inositol was not administered to similar animals, death resulted after their weight had decreased to 15 to 20 gm. Death was averted only when inositol was fed soon after the loss of weight became apparent. In several cases these phenomena were not accompanied by alopecia at any stage of the experiment; and in no case was loss of hair observed during the period of precipitous loss of weight. Thus inositol deficiency was not invariably accompanied by alopecia.

The low incidence of alopecia has been reported on previous occasions (4). Examination of the data for individual mice in the first experiment described above demonstrated again that inositol deficiency cannot be produced in all animals. In contrast to the uniformity in content of inositol of weanling mice (Table I) it was found that several of the animals kept on the inositol-free ration had a low content. Thus it was low in two out of the ten analyzed after 2 weeks (0.24 and 0.27 gamma per mg., as compared to 0.35 for the average of the group); and after 4 weeks in three out of ten (0.30, 0.29, 0.31 gamma per mg.). In an independent group of seven mice three showed precipitous loss of weight after 7 weeks; in a group of five mice two behaved similarly; while in a third group of seven two lost weight as described.

Relation of Pantothenic Acid to the Synthesis of Inositol.—In order to investigate more fully the relationship of pantothenic acid and inositol to alopecia (4) the following experiments were performed. A group of twenty-two weanling mice were fed the above ration, minus pantothenic acid. Ten animals were analyzed at the beginning of the experiments. The remaining twelve were analyzed 3 weeks later. The average content of inositol in the first group was the same as that previously recorded in Table II for weanling mice (0.49 gamma per mg.; 4.0 mg. per mouse). The average values in the second group were 0.24 gamma per mg. and 4.3 mg. per mouse. Thus the inositol content per unit weight decreased and the total amount of this substance in the mouse remained approximately the same.

The experiment was repeated twice with groups of six mice with the same result as in the first trial. In a fourth experiment with 12 mice the content of inositol did not decrease significantly (0.48 to 0.41 gamma per mg.) in 2 weeks, undoubtedly because of the small gain in weight of the group (from 6.5 gm. to 10 gm.). That is, the quantity of inositol contained in a mouse at the start and presumably carried through the test period was not distributed in as much tissue as in the other experiments.

Addition of pantothenic acid to the diet of animals deficient in this vitamin resulted in the synthesis of inositol. A typical experiment was as follows:

A group of fifteen mice were fed the ration deficient in pantothenic acid for 2 weeks. The average content of inositol at the beginning was 0.49 gamma per mg. and the total amount in the average mouse was 4 mg. After the mice had been deficient in pantothenic acid for 2 weeks the content of inositol had decreased, but the total amount in a mouse was the same as at the beginning (judged from the average of two mice analyzed). Pantothenic acid was then restored to the diet. Two to 3 weeks after the restoration the typical alopecia of inositol deficiency made its appearance in seven of the mice. Four out of the seven hairless individuals exhibited spontaneous cure of the alopecia 2 to 3 weeks after its appearance even though no inositol was fed. Analyses of these spontaneously cured animals revealed that in them inositol had increased to normal values (0.39 gamma per mg.; 11.7 mg. per mouse). Analyses of those which remained hairless showed that they had not increased markedly in content of inositol.

Microbial Synthesis of Inositol

In an effort to discover the reason for the spontaneous cures of alopecia which were observed (4, and above) the following experiments were performed:

The intestinal tract of a mouse which had exhibited spontaneous cure of alopecia was removed aseptically and placed in 10 cc. of a synthetic medium. This medium had the composition described by Woolley (5), except that inositol and thioglycollic acid were omitted. It was thus a highly purified mixture of all available growth factors and of glucose, inorganic salts, and amino acids. The tube was incubated for 24 hours at 37°, and then a drop of the suspension was introduced into a second 10 cc. of medium of the same composition. After 24 hours incubation, 1 cc. of this passage of culture of organisms from the intestinal tract was added to 500 cc. of medium of the same composition. Incubation at 37° was continued for 60 hours. The cells were then collected by centrifugation, hydrolyzed, and analyzed for inositol. The metabolism solution was concentrated under reduced pressure and similarly analyzed.

As is shown in Table III, the organisms obtained by passage of a culture from the intestinal tract synthesized inositol and at least 80 per cent of the amount formed was retained in the cells. The experiment was repeated with the same mixed culture (which had been stored at 0°) for an incubation period of only 16 hours. Approximately the same quantity of inositol was formed as previously. Hence the incubation periods in subsequent tests were 16 hours in length.

The intestinal tract of a mouse from the same group of animals which had lost its hair and had not exhibited spontaneous cure was treated similarly. The bacteria from this mouse showed a much smaller content of inositol (Table III). The experiments were repeated on two mice from a second run, one of which had become hairless but had then exhibited a spontaneous cure and the other of which had remained hairless. The culture of organisms from the first mouse synthesized 0.42 gamma of inositol per cc. of culture and that from the second mouse formed 0.12 gamma. A third experiment with two more mice, cured and hairless like the others, was performed in the same manner and it was found that the values were 0.40 and 0.0 gamma per cc. for the

culture from the spontaneously cured mouse and the hairless mouse respectively. In this case the hairless mouse was examined as soon as alopecia developed, while in the other instances animals were taken which had been hairless for about one week.

It has been found that the synthesis by the cells of the culture was not influenced by gramicidin and that a prominent intestinal inhabitant did not form inositol. One of the cultures already tested, which was procured from a mouse which had exhibited spontaneous cure of alopecia, was inoculated into a medium of the composition described above, to which had been added 10 gamma per cc. of crystalline gramicidin.[1] A control test was done, using the same culture without the addition of gramicidin. The cells from each medium were collected and analyzed for inositol. As can be seen from Table III gramicidin did not

TABLE III

Inositol Content of Cultures of Intestinal Organisms

The numbers in parentheses indicate the length of the period of incubation.

Material analyzed	Inositol content of culture
	gamma/cc.
Cells of culture from spontaneously cured mouse (60 hrs.)	0.40
Supernatant fluid after centrifugation of the above cells	Less than 0.1
Cells of mixed culture from above mouse (16 hrs.)	0.38
Cells from the above culture grown in gramicidin	0.38
Cells of mixed culture from hairless mouse	0.18
Cells of *E. coli*	Less than 0.1

influence the synthesis. The cells of one of the most important bacterial forms in the intestinal tract of the mouse, *Escherichia coli*, as grown in the purified medium, were analyzed and found not to contain inositol in demonstrable amounts (Table III).

Influence of Pantothenic Acid on Inositol Metabolism

The occurrence of alopecia in a high percentage of the mice to which pantothenic acid had been restored after 2 or 3 weeks of deficiency has been mentioned above. It seemed likely that if inositol were fed from the beginning, this delayed appearance of alopecia might be prevented. A group of ten mice were fed the basal ration referred to throughout this paper, from which pantothenic acid was omitted and which was supplemented with 100 mg. of inositol per 100 gm. After 2 weeks pantothenic acid was restored and inositol was omitted. Two to $2\frac{1}{2}$ weeks after this change typical alopecia developed in all but three of the animals. Analysis of three of the hairless mice showed that they contained little more inositol than did weanling mice (6.4 mg. compared to 4.5 mg.).

[1] We wish to thank Dr. R. J. Dubos for gifts of gramicidin.

Similarly, it had been found that two of the animals at the time that pantothenic acid was added were deficient in inositol even though they had received it in the diet (4.0 mg. compared to 4.4 mg.).

The experiment was repeated with the following modifications. A group of six mice were fed the ration which contained inositol but no pantothenic acid. After 2 weeks analysis of three mice again showed that they were deficient in inositol. The other three mice were continued on inositol for 3 days following the addition of pantothenic acid. Analyses of these revealed a normal content of the vitamin (0.42 gamma per mg.). Thus it appeared that when pantothenic acid was absent from the ration inositol deficiency developed even though this substance was ingested. Addition of pantothenic acid to the diet restored the inositol content of the mice to normal within 3 days.

TABLE IV

Distribution of Free and Total Inositol in Various Natural Products

Except in the case of the extracts analyses were based on weights of undried samples.

Material analyzed	Free inositol	Total inositol
	gamma/mg.	*gamma/mg.*
Normal mice (6 individuals)	0.25	0.41
Inositol-deficient mice (3 individuals)	0.20	0.30
Beef skeletal muscle	0.55	0.88
Beef brain	3.0	6.0
Beef pancreas extract	1.2	5.0
Aqueous alcohol extract of rice bran	0.37	0.53
Dialyzed rice bran extract	0.0	0.16

Free and Combined Inositol

Since it has been shown (6) that yeast is unable to respond to inositol esters, it was thought possible that the method for the estimation might be refined to differentiate free from combined inositol. It has been shown (2) that liver contains a non-dialyzable, water-soluble substance which liberates inositol when treated with acid or alkali. It has been found that yeast does not respond to this combination. This has made possible the analysis of natural products for free inositol, separate from total inositol. The difference between free and total represents combined inositol.

For the analysis of mice for free inositol the following procedure was used.

A mouse was ground and suspended in water. An aliquot of this suspension was hydrolyzed with HCl and total inositol was determined in the hydrolysate as previously described. A second aliquot of the suspension was heated in an autoclave (15 pounds for 15 minutes), centrifuged, and the precipitate washed with water. Direct analysis of this liquid without acid hydrolysis gave a value for free inositol. Since the combined inositol present in liver extract, brain extract, pancreas extract, and rice

bran extract was not rendered available to yeast by autoclaving, this procedure was used to coagulate proteins and destroy enzymes.

By this technique approximately 60 per cent of the inositol content of normal mice was found to be free. Similarly, 50 per cent of the total inositol content of brain and 60 per cent of that in skeletal muscle was found to be free. The proportion of free inositol in normal mice did not differ significantly from that in deficient animals. Some representative data are shown in Table IV. In the case of tissues the value for free inositol represents the amount which can be extracted and does not include any free inositol which may be retained in insoluble residues.

DISCUSSION

It can be clearly seen from the data in Table II that mice synthesized inositol when fed the purified ration. It appears that the presence of pantothenic acid in the ration was of importance for this synthesis. Those animals which did not receive pantothenic acid failed to increase markedly in total inositol content even though they did gain in weight; whereas, when pantothenic acid was added to the ration, the inositol content of the mice increased. It may be of interest to note that in no case of deficiency did the total amount of inositol in a mouse decrease even though the content per unit weight did fall. Hence it was necessary that the animals should grow if the inositol deficiency was to be recognized by a decreased content of the vitamin per unit weight.

One site of inositol synthesis appears to be the intestinal tract. When a mouse exhibited spontaneous cure of alopecia, a simultaneous increase in the total content of inositol took place. Organisms cultivated from the intestinal tract of mice which had exhibited spontaneous cure synthesized inositol in the instances tested and to a much greater extent than did the organisms isolated by the same method of cultivation from hairless mice. Since the synthesis took place in the presence of gramicidin, which inhibits the growth of Gram-positive bacteria (7) and since the gramicidin-treated cultures on microscopic examination were found to be Gram-negative, it is probable that the organisms responsible for the synthesis were Gram-negative. However, a prominent Gram-negative organism of the intestinal tract, *E. coli*, did not form inositol. Since inositol deficiency could be produced regularly in a small percentage of mice fed a deficient diet, it must be concluded that the inositol synthesized by such organisms was not sufficient to meet all of the requirements of the mouse, or else that, under the conditions studied, the organisms did not become established in all animals. This latter hypothesis may explain more adequately why only some animals on an inositol-free diet develop alopecia. The data show further that the metabolism of inositol is influenced by pantothenic acid. Mice did not increase in total content of inositol even when this sub-

stance was present in the diet unless pantothenic acid was also present. It has not been established whether this phenomenon was due to failure of absorption or to some more obscure metabolic disturbance.

SUMMARY

It has been shown that mice are able to synthesize inositol. This synthesis was not observed when pantothenic acid was absent from the diet. Cultures from the intestinal tract of animals which exhibited spontaneous cure of alopecia yielded microorganisms which synthesized much more inositol than did organisms isolated in the same fashion from the tracts of mice that had become hairless. Some observations on the distribution of free and combined inositol have been made and it has been shown that several biological materials contain combined inositol. It has been found that deficiency of inositol can develop even when inositol is present in the diet if pantothenic acid is omitted.

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