

EXPERIMENTAL MEGALOBLASTIC ANEMIA AND SCURVY IN THE MONKEY

V. NATURE OF THE RELATION OF ASCORBIC ACID DEFICIENCY TO THE METABOLISM OF FOLIC ACID COMPOUNDS¹

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ONE FIGURE

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Once it was found that a deficiency of folic acid compounds and megaloblastic anemia developed regularly in scorbutic monkeys (May et al., '51, '52a), it became interesting to determine what influence ascorbic acid deficiency had on the metabolism of folic acid compounds.

The experiments described in this report were devised to answer the question: Does ascorbic acid deficiency disrupt the normal metabolism of folic acid at one or more points, or do some non-specific factors operating in scurvy increase the requirements for folic acid? To this end a systematic examination of the effects of ascorbic acid deficiency on each of the known phases in the metabolism of folic acid compounds was undertaken.

Present understanding of the metabolism of folic acid compounds may be outlined as follows (Welch and Heinle, '51): Primates do not appear to synthesize folic acid compounds

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but obtain them from the diet or from the synthetic activities of the bacteria in the intestine. In both instances the folic acid usually occurs almost entirely in a conjugated form, a folic acid heptaglutamate. Presumably the folic acid must be liberated from the conjugate prior to utilization in metabolic reactions. This is accomplished by conjugases in the tissues, notably the kidney and liver. Folic acid can be converted by enzymes in the liver to a derivative, folinic acid (citrovorum factor), which may be the biologically active form. Folic acid or folinic acid plays a role in the synthesis of nucleic acids essential for normal hematopoiesis. Some workers are of the opinion that folinic acid and its conjugate are the naturally occurring forms and that folic acid is merely produced by alterations during isolation. This consideration does not require any modification of the present outline.

Consequently, it was necessary to test the effect of ascorbic acid deficiency on: (1) the bacterial synthesis of folic acid compounds, (2) the absorption of the conjugate from the intestine, (3) the liberation of the folic acid by the action of the conjugase, (4) the conversion of folic acid to folinic acid, and (5) the comparative activity of folic acid and folinic acid on megaloblastosis in scurvy.

ARRANGEMENT OF EXPERIMENTS AND METHODS

The general management, diets, and descriptions of the monkeys used in the experiments were as previously described (May et al., '51, '52a). The methods used in microbioassay of the folic acid compounds have also been published (May et al., '52a). Free folic acid (PGA) was determined in the specimens with *S. fecalis* after autoclaving, and total PGA after subsequent treatment with hog kidney conjugase. Free folinic acid (FNA) was determined with *L. citrovorum* after autoclaving the specimens without enzyme treatment. It will be recalled that "total PGA" as determined with *S. fecalis* includes folinic acid compounds as well as folic acid compounds. Free FNA may be a more accurate measure of biologically active material.

RESULTS

In the previous paper data were reported on the PGA in the diet and feces (May et al., '52a). The milk diet fed the monkeys was found to contain 2.6 μg total PGA per liter. The control animals consumed 400 to 600 ml a day, making the intake of total PGA approximately 1 μg a day. The most anorexic of the scorbutic animals drank 100 ml daily, leading to a reduction of PGA intake to about one-fourth. The PGA in milk may be predominantly in the free form (Hodson, '49).

The average daily fecal excretion of total PGA was found to be the same in the scorbutic monkeys as in the controls, about 29 μg a day. The PGA was almost entirely in the conjugated form in both cases. This indicates that ascorbic acid deficiency probably did not affect the bacterial synthesis of folic acid compounds in the intestines of these monkeys.

*Absorption and utilization of folic
heptaglutamate*

The extent to which the conjugated PGA in the feces is available to the animals can be estimated from the following experiments:

To compare the absorption and utilization of folic acid compounds, equal amounts of folic acid in the free and conjugated forms were given to control and scorbutic monkeys, orally and intramuscularly, and the amount of free folic acid excreted in the urine was measured. It was found, by treating the urine with conjugase, that conjugated folic acid was not excreted as such in the urine even when injected intramuscularly, by either the control or the scorbutic monkeys. The data presented in table 1 indicate that orally administered free and conjugated PGA are absorbed equally well by the scorbutic and the control animals and that probably conjugated PGA is poorly absorbed by both.

The liberation of PGA from the heptaglutamate was performed normally by the scorbutic animal, as judged by the appearance of 80% or more of the intramuscularly administered conjugated PGA as free PGA in the urine. This was

TABLE 1

Utilization of folic acid (PGA) and folic heptaglutamate (PGAC) by scorbutic and control monkeys

ANIMAL NO.	DIET	EXCRETED IN URINE WITHIN 24 HOURS:		%	
		As free PGA	As conjugated PGA		
<i>Controls (50 mg ascorbic acid daily)</i>					
39	4B	500 μ g PGA by mouth	17
30	4B	500 μ g PGA intramuscularly	88
37	4B	500 μ g PGA intramuscularly and 15 μ g B ₁₂ intramuscularly	86
142	4F	200 μ g conjugated PGA (75 mg PGAC concentrate ¹) by mouth	2		None ²
142	4F	200 μ g conjugated PGA (75 mg PGAC concentrate ¹) intramuscularly	39		None
<i>Scorbutic</i>					
127	4D	200 μ g PGA b.m.	24		None
128	4D	200 μ g conjugated PGA (75 mg PGAC concentrate ¹) by mouth	4		None
129	4D	200 μ g conjugated PGA (75 mg PGAC concentrate ¹) intramuscularly	83		None

¹ A concentrate of folic acid heptaglutamate derived from yeast (Greene, '49) and supplied through the courtesy of Dr. Richard D. Greene, E. R. Squibb and Sons. Assayed in our laboratory to contain 0.79% heptaglutamate. Seventy-five milligrams of this concentrate provided the equivalent of 200 μ g of folic acid activity as determined by treatment with conjugase and assay with *S. fecalis*.

² No increase in free PGA was found in the urine after treatment with conjugase.

substantiated by the finding of: (1) the same ratio of free to total PGA in the livers of control and ascorbic acid-deficient monkeys (table 2) and, (2) that liver from scorbutic monkeys

TABLE 2

Free vs. total PGA in livers of monkeys
(Micrograms per gram, wet)

CONDITION OF MONKEYS	AVERAGE TOTAL PGA	AVERAGE FREE PGA
Adequate ascorbic acid	0.60	0.27 (45%)
Ascorbic acid deficiency	0.13	0.07 (54%)

TABLE 3

In vitro folic acid conjugase activity of monkey livers

TREATMENT OF YEAST EXTRACT ¹	μG FOLIC ACID LIBERATED PER GRAM DRIED YEAST
No treatment (free PGA)	0.6
Hog kidney "conjugase" (total PGA)	25
Control monkey liver "conjugase"	23
Scorbutic monkey liver "conjugase"	20

¹ *Preparation of yeast extract:*

Five grams of dried brewers' yeast were suspended in 50 ml distilled water, adjusted to pH 6.5, autoclaved 5 minutes at 15 pounds, cooled, centrifuged, and the supernatant solution was made up to 100 ml.

Preparation of hog kidney and monkey liver "conjugase":

The tissue was passed through a tissue press and weighed. Three parts of distilled water were added and the suspension homogenized in a Potter-Elvehjem homogenizer.

Procedure:

To 5 ml of yeast extract were added 5 ml of McIlvaine's phosphate buffer (pH 4.5) and 1 ml of the "conjugase" preparation to be tested for activity. The well-mixed suspension was then incubated for 20 hours at 37°C., placed in a boiling water bath for two minutes, neutralized with 10% NaOH, made up to a volume of 20 ml with distilled water, filtered, and diluted for assay with *S. fecalis*.

was almost as effective a source of conjugase for liberating PGA from the conjugated form in yeast as was the standard hog kidney conjugase preparation or liver from a control monkey (table 3).

Conversion of PGA to FNA

An attempt was made to ascertain the efficiency of conversion of folic acid to folinic acid by measuring the amount of free FNA excreted in the urine following an intramuscular test dose of free PGA (40 μ g per kilogram). The concentration of FNA excreted by both the control and the scorbutic monkeys was too small for accurate analysis, although 25% of the administered PGA was recovered from the urine.

Another approach to detecting any effect of ascorbic acid deficiency on the metabolism of folic acid compounds, including the conversion of PGA to FNA, was to compare the accumulation of these compounds in the liver with the effect on the megaloblastic marrow when free or conjugated folic acid was administered to scorbutic megaloblastic monkeys, orally or intramuscularly. Data from this study are presented in table 4. The following considerations should be employed in evaluating the data in this table: The scorbutic megaloblastic animals were selected to be as similar as possible, so that the liver stores and marrows would be in nearly the same state before treatment. They were grouped so that test doses were given in equal amounts for the same length of time before comparing the marrows and killing the animals for liver assays. Animals to be compared were treated simultaneously with the same solutions of test materials. The diets were essentially the same, thus keeping the trace of PGA compounds obtainable from this source at a constant level.

It is evident that the scorbutic animal accumulated FNA in the liver after small doses of PGA, free or conjugated, with corresponding alleviation of the megaloblastosis in the marrow, without the aid of ascorbic acid (note animals 92 and 123). Also it may be seen that larger doses are required orally than intramuscularly. (Compare animals 120 and 119 with 92 in the case of free PGA, and 122 with 123 in the case of conjugated PGA.) The better absorption of orally administered free PGA versus conjugated PGA is not so clearly demonstrated as in table 1. It appears that scorbutic

TABLE 4

Effect of folic acid (PGA) and folic heptaglutamate (PGAC) on the megaloblastic marrow and the liver content of PGA and free folic acid (FNA) in scorbutic monkeys¹

	ANIMAL NO.	DIET	B ₁₂	TOTAL PGA	FREE FNA	
				$\mu\text{g/gm wet liver}$		
<i>Untreated</i>						
Controls — normoblastic	Averages ²	4B,4F	1.0	1.10	0.026	
Scorbutic — megaloblastic	Averages ²	4C ²	1.0	0.20	0.010	
Scorbutic — megaloblastic	Averages ²	4F	0.5	0.12	0.004	
<i>Treated 12 days:</i>						
100 μg PGA by mouth	120	4F	0.5	0.24	0.009	Marrow after treatment
100 μg PGA intramuscularly	92	4C ²	1.3	0.39	0.033	Equivocal effect
200 μg PGA b.m.	119	4F	0.5	0.47	0.021	Normoblastic
100 μg conjugated PGA ³ b.m.	122	4F	0.6	0.34	0.013	Megaloblastic
100 μg conjugated PGA i.m.	123	4F	0.6	0.38	0.051	Normoblastic
200 μg conjugated PGA b.m.	124	4F	0.5	0.37	0.013	Megaloblastic
200 μg conjugated PGA i.m.	117	4F	0.5	0.24	0.020	Normoblastic
<i>Treated 7 days:</i>						
200 μg PGA b.m.	127	4D	0.5	0.40	0.017	Normoblastic
200 μg conjugated PGA b.m.	128	4D	0.5	0.17	0.029	Equivocal effect
200 μg conjugated PGA i.m.	129	4D	0.5	0.18	0.018	Normoblastic
<i>Treated prophylactically:</i>						
500 μg PGA b.m. weekly	39 ⁴	4B	0.6	1.69	Normoblastic
500 μg PGA i.m. weekly	30 ⁴	4B	0.4	1.05	Normoblastic
500 μg PGA } 15 μg B ₁₂ } i.m. weekly	37 ⁴	4B	1.0	1.35	Normoblastic

¹ The monkeys were allowed to reach essentially the same degree of scurvy and megaloblastosis, treated for the same lengths of time as indicated, and then, after examining the marrow, the animals were killed and the livers assayed for comparison of the accumulations of PGA and FNA.

² Average values from previous report (May et al., '52a). Diet 4C was supplemented with intramuscular injections of vitamin B₁₂; all the other diets had no supplementary B₁₂ or the animals received a supplement orally. Vitamin B₁₂ neither prevents nor cures the megaloblastosis complicating scurvy. Controls received an abundance of ascorbic acid.

³ A concentrate of folic acid heptaglutamate derived from yeast (Greene, '49) and supplied through the courtesy of Dr. Richard D. Greene, E. R. Squibb and Sons. Assayed in our laboratory to contain 0.79% heptaglutamate. Seventy-five milligrams of this concentrate provided the equivalent of 200 μg of folic acid activity, as determined by treatment with conjugase and assay with *S. fecalis*.

⁴ Killed after signs of scurvy had been evident for three to 4 weeks.

animals absorb conjugated PGA poorly (note animals 122, 124) but no more so than control animals (see table 1). It was not possible on the basis of these data to determine whether ascorbic acid might augment the conversion of PGA to FNA *in vivo* in the monkey, as Nichol and Welch ('50) have reported it did during *in vitro* experiments with rat liver slices. It can only be asserted that an abundance of ascorbic acid does not appear to be necessary for the conversion of PGA to FNA *in vivo* in the monkey. In other studies serial observations of the marrow showed the rate of recovery from megaloblastosis to be equally rapid in scorbutic animals following large doses of PGA or ascorbic acid (Sundberg et al., '52).

Comparative activity of folic and folinic acids on megaloblastosis in scurvy

That synthetic folinic acid does not require ascorbic acid for its action has already been reported in preliminary form (May et al., '50b). Figure 1 presents data from that study in graphic form to show that the rate of disappearance of megaloblasts from the marrows of scorbutic monkeys was as rapid after 7.5- μ g doses of folinic acid as it was after 750- μ g doses of folic acid, and that 50- μ g doses of folic acid were unable to halt the progression of the megaloblastic process. This might be interpreted as indicating that the conversion of PGA to FNA is inefficient in scurvy if FNA is considered as the biologically active form. Scurvy is the only condition in which a defective conversion of PGA to FNA has been suggested, and provides the best circumstances in which the relative biologic potency of PGA and FNA might be compared more extensively.

DISCUSSION

On the basis of these studies, the conversion of PGA to FNA is the only area in which a deficiency of ascorbic acid might be considered to affect the metabolism of folic acid

compounds. All other phases in the metabolism of these compounds were found to be unaffected in scurvy. It has been pointed out that whereas the efficiency of conversion of PGA to FNA may be impaired in scurvy, the evidence for this in these studies in the monkey was scanty. The conversion proceeded sufficiently well in scurvy to permit small doses

Experimental Megaloblastic Anemia
Effects of Folic ■ and Folinic ▨ Acids on the Marrow

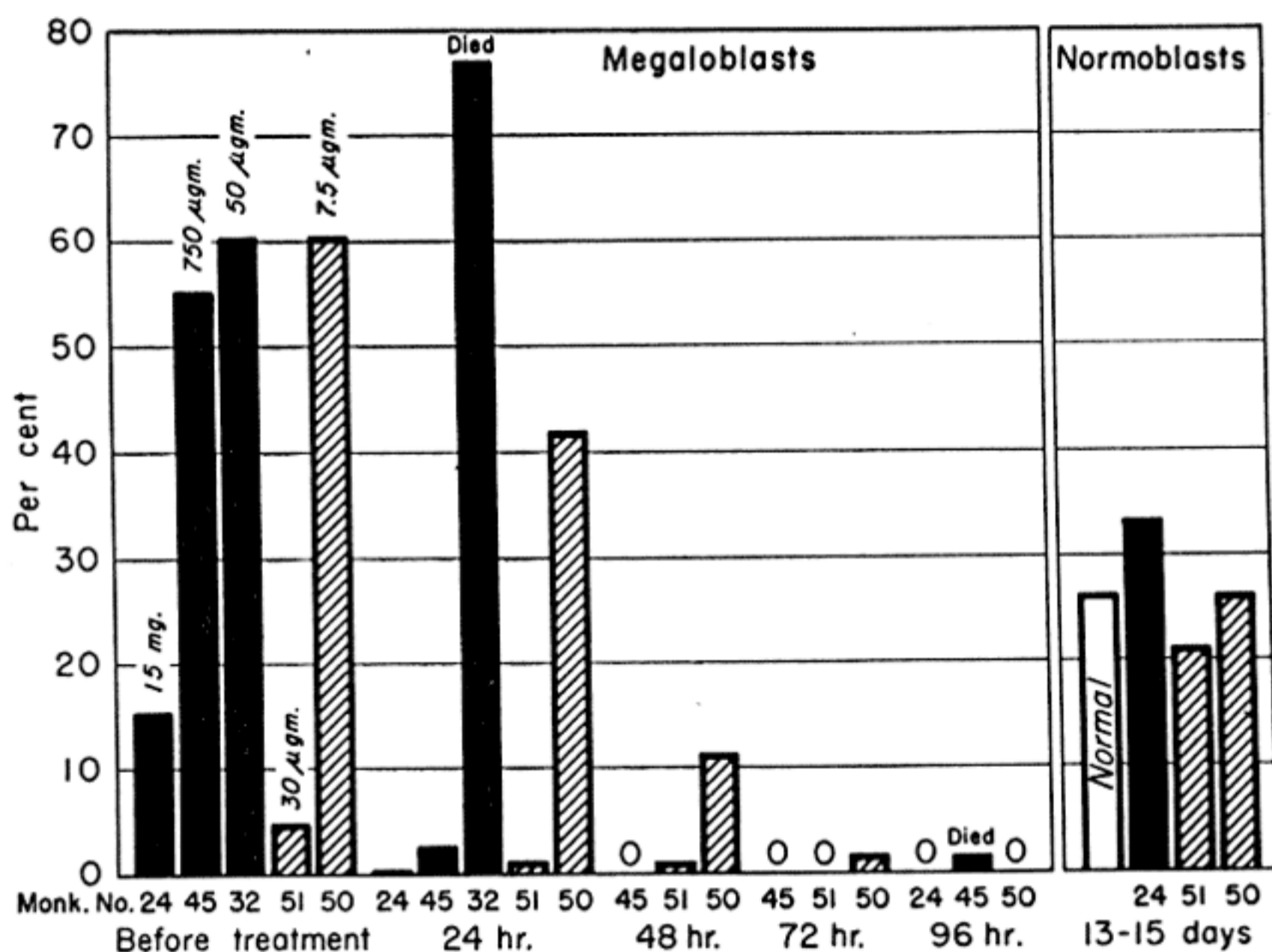


Fig. 1 Comparison of effects of folic and folinic acids on megaloblastic marrows in scorbutic monkeys. (From data in study by May, Sundberg and Schaar, '50b). The symbol O indicates that the marrow was not examined at the corresponding time interval.

of PGA to be used effectively for correction of the deficiency of FNA and elimination of the megaloblastosis.

It therefore seems necessary to seek an explanation other than ascorbic acid deficiency to account for the development of so profound a deficiency of PGA and FNA as occurs in scurvy. It would seem more reasonable to attribute this de-

iciency to inability to meet an increased requirement for PGA in scurvy, due to greater use or wastage of PGA because of non-specific factors operating in scurvy. This may be expressed as follows:

Increased daily requirement for PGA in scorbutic monkeys on milk diets

Potentially available PGA:

1. Milk diet, 2 μg free PGA.
2. Feces, 30 μg conjugated PGA which may be poorly absorbed.

With this intake:

Controls, ascorbic acid-adequate, do not develop PGA deficiency. Scorbutics develop PGA deficiency. Supplements of 5 μg of free PGA daily cannot prevent deficiency (May et al., '50a), but 70 μg daily will prevent deficiency (animals 30, 37, 39, table 4).

Thus the scorbutic monkeys could not obtain enough PGA from the milk diet and their feces to maintain the tissue stores at a normal level, but required a supplement of PGA to prevent deficiency.

It is also pertinent to recall that PGA deficiency sufficiently severe to lead to megaloblastosis rarely developed until signs of scurvy had been manifest in the monkey for about two weeks (May et al., '51). It was not merely a deficiency of ascorbic acid alone, but actual clinical scurvy with its attendant tissue hemorrhages and pathology, which caused the difficulty. It can be seen in table 5 that the stores of ascorbic acid declined rapidly on the deficient diet, reaching a level nearly as low as that found in scurvy in about 30 days, though scurvy did not appear for another 50 to 60 days. The levels of folic acid compounds fell more gradually until signs of scurvy appeared, and then a sharp decrease in PGA and particularly in FNA occurred.

This raises the question: What causes the sharp decline of PGA compounds in the tissues upon the advent of scurvy? At present we suspect that scurvy is complicated by a defi-

ciency of PGA and FNA and by megaloblastosis primarily because of non-specific factors in scurvy which cause increased use or loss of PGA, rather than because of any specific role of ascorbic acid in the metabolism of folic acid compounds. The diffuse pathology of scurvy may be considered a form of "stress." The effect of "stress" situations on the tissue stores of folic acid compounds has been under investigation (May et al., '52b); infection causes marked depletion of tissue stores of folic acid compounds, even when the concentration of ascorbic acid in the tissues is normal.

TABLE 5

Relation between development of ascorbic acid deficiency and folic acid deficiency

ANIMALS	ASCORBIC ACID	TOTAL PGA	FREE FNA	MARROW
	<i>μg/gm wet liver</i>			
<i>Controls (average)</i> ¹	129	1.10	0.026	Normoblastic
<i>No ascorbic acid</i>				
30 days (No. 157) biopsy	12	0.96	0.018	Normoblastic
82 days (No. 157) biopsy	6	0.52	0.023	Normoblastic
101 days (No. 157) scurvy 7 days	4	0.23	0.003	Megaloblastic

¹ From tables 2 and 4 in previous paper (May et al., '52a).

It is possible that vitamin B₁₂ is involved in the metabolism of folic acid compounds. Our scorbutic monkeys were not markedly deficient in vitamin B₁₂. If vitamin B₁₂ requires ascorbic acid to function normally, then the metabolism of folic acid compounds might be disturbed in scurvy in this indirect manner. A suggestion to an obstetrical colleague, that B₁₂ might be more effective in the megaloblastic anemia of pregnancy if ascorbic acid were given first, led to the finding that this was the case (Holly, '51). The interrelations of B₁₂, ascorbic acid and folic acid will have to be studied by means of animals made deficient in B₁₂ as well as ascorbic acid.

CONCLUSIONS

As a result of a systematic study of the effect of ascorbic acid deficiency on the metabolism of folic acid compounds it was concluded that:

1. The conversion of folic acid to folinic acid *in vivo* may be less efficient in scurvy, but ascorbic acid is not necessary for this conversion to take place. All other phases of the metabolism of folic acid compounds tested were found to proceed normally in scurvy; ascorbic acid is not required.

2. Ascorbic acid deficiency was not accompanied by a marked deficiency of folic acid compounds until signs of scurvy had become well advanced.

3. The severe deficiency of folic acid compounds which occurred regularly as a complication of scurvy in monkeys fed milk diets was probably due to non-specific factors operating in scurvy.

4. The net effect of scurvy was to cause increased requirements for folic acid compounds which could not be met by the supply of these compounds obtainable from the milk diet and the feces.

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