THE INFLUENCE OF BIOTIN UPON SUSCEPTIBILITY TO MALARIA

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(Received for publication, March 31, 1943)

That individuals differ in their degree of susceptibility to malaria has long been an accepted fact. Such differences have been ascribed to many and varied factors, but it has never been possible to demonstrate a direct relationship between any particular factor and the degree of susceptibility. Nutritional status has received special emphasis in this connection, and it is said that "nutrition is of the greatest importance in the reaction to malaria" (1). There has been, however, but scant clinical or epidemiological evidence, and no experimental evidence, in favor of this idea. While it is true that malnutrition and famine frequently accompany severe epidemics of malaria, it is as likely that the malaria brings about the famine as that the famine makes the malaria more severe. Moreover, as pointed out by Russell (2), famine conditions may indirectly increase the severity of a malaria epidemic by causing malarious individuals to attempt to work before they are fit, increasing the relapse rate, or by driving infected families into other areas, which thus become seeded with gametocyte carriers. The only experimental data on the subject are those of Passmore and Sommerville (3). They found that *Macacus radiatus* monkeys, kept on a diet similar to that of the rice-eating poor of India and especially deficient in vitamins A and C and in calcium, did not develop more severe infections with the monkey malaria parasites *Plasmodium cynomolgi* and *P. knowlesi* than did control monkeys on an adequate diet.

As we have already reported in a preliminary way (4), experiments with avian malaria have now shown that the level in the host animal of biotin (5), an essential growth factor, influences greatly the severity of the infection. Moreover, the level of biotin in the blood of chickens and ducks infected with *Plasmodium lophurae* has been found to increase during the course of the acute infection and to return to normal when the infection subsides.

Methods

Young Rhode Island Red chickens and White Pekin ducks were rendered biotin-deficient by feeding them a diet containing a large proportion of dried egg white (6). In the simplest type of experiment, the control animals, which were of the same breed, age, and group as the deficient ones, received the same diet with the egg white replaced by casein. In other experiments the control animals were fed the egg white diet, but they received biotin concentrate by mouth or by intraperitoneal injections. When

the animals were 2 to 4 months old and those on unsupplemented egg white diet showed signs of biotin deficiency, they were all inoculated intravenously with appropriate doses, proportional to their body weight, of malaria parasites.

Plasmodium lophurae (7) was used for most of the experiments. This parasite, if inoculated intravenously in sufficiently large numbers, produces very heavy infections in ducks (8, 9) and in baby chicks, but only mild infections in older chickens (7, 10–12). The experimental animals were inoculated with an amount of heparinized blood from a heavily infected chicken or duck sufficiently large to permit an accurate parasite count to be made on a thin blood film prepared immediately after inoculation. The number of parasites per 10,000 red blood cells was determined in the usual manner (13) on the initial blood films and on blood films prepared daily beginning with the 2nd or 3rd day and continuing through the 5th, 6th, 7th, or 8th day after inoculation.

Since relative number of parasites was used as the measure of intensity of infections, it was obviously important to be certain that the blood volume and red blood cells per cubic millimeter at the time of inoculation were approximately the same in experimental and control animals. Direct measurements showed that ducks kept on egg white diet for 2 weeks had the same amount of red blood cells per cubic millimeter as those not deficient in biotin. Similarly, chicks which were biotin-deficient after more than 4 weeks on egg white diet had a hemoglobin of 85 per cent by the Tallquist method, just as did the control chicks on the casein diet. The initial parasite counts themselves, made in every experiment from blood films prepared immediately after inoculation of the animals, provide the most conclusive evidence that the proportion of red blood cells and the blood volume in relation to body weight were entirely comparable in biotin-deficient and non-deficient animals. If, for example, the biotin-deficient animals had been, at the time of inoculation, anemic as compared to the non-deficient animals, then the injection into them of doses of parasites at the same rate, in proportion to body weight, as for non-deficient animals would have yielded higher initial relative parasite counts for the deficient than for the non-deficient animals. Actually, the average initial parasite densities for the different groups of animals in each experiment were remarkably uniform. (See, for example, Tables I and II, Charts 1 to 5.)

Biotin assays were made on the blood of some of the animals before inoculation and during the course of the infection.

Diets.—The animals to be made biotin-deficient and the control animals were supplied daily with equivalent amounts of food, and usually most of it was consumed by the next day. The following diets were used (composition given in percentage by weight).

A: A regular baby chick mash. Yellow corn meal 25.5, wheat bran 8.5, wheat middlings 8.5, ground wheat 17.1, pinhead oats 17.1, soybean oil meal 2.6, meat scrap 6.0, skim milk 4.3, alfalfa leaf meal 8.5, limestone flour 1.2, fortified cod liver oil 0.3, salt 0.4.

B: A standard duck food. Yellow corn meal 25, wheat bran 25, wheat middlings 25, meat scrap 25. Stock ducks received this diet as a moist mash mixed with a little chopped lettuce. When this diet was used in experiments, it was fed dry without any lettuce.

1: Yellow corn meal 40, wheat middlings 25, bran 10, powdered egg white 25, plus a small amount of grits and oyster shell.

1*a:* Same as 1, but egg white replaced by washed casein.

2: (After (6)). Yellow corn meal 48, wheat middlings 24, powdered egg white 24, bone meal 1.5, cod liver oil 1, salt 0.5, ground oyster shell 1.

2*a:* Same as 2, but egg white replaced by washed casein.

3: Diet B 75, powdered egg white 25.

3*a:* Same as 3, but egg white replaced by washed casein.

3*b:* Same as 3*a*, but casein first mixed with riboflavin to provide 5 mg. riboflavin per 100 gm. casein.

4: Diet A 75, powdered egg white 25.

4*a:* Same as 4, but egg white replaced by washed casein.

4*b:* Same as 4*a*, but casein first mixed with riboflavin to provide 5 mg. riboflavin per 100 gm. casein.

4-I: Diet 4 mixed with enough biotin concentrate (S.M.A. Co. No. 1000) to supply 500 γ per kg. of food in addition to the biotin of the food. This left little excess biotin over that which combined with the egg white.

4-II: Diet 4 mixed with enough biotin concentrate to supply 1 mg. biotin per kg. of food in addition to the biotin in diet 4. This left an excess, over that which combined with the egg white, of about 40 γ per 100 gm. of diet.

5: (After (14)). Yellow corn meal 58 parts, wheat middlings 25 parts, washed casein 12 parts. These three ingredients were mixed and heated in shallow layers in an oven at 120°C. for 30 hours. They were then mixed with one part each of $CaCO_3$, $CaHPO_4 \cdot 2H_2O$, and NaCl, and with the following amounts of vitamins per kg. of ration: thiamin 1 mg., riboflavin 1 mg., 2-methyl-1,4-naphthoquinone 10 mg.

5-I: Diet 5 supplemented with 15 mg. calcium pantothenate per kg. (15).

Animals on diets 3, 3*a*, 3*b*, 4, 4*a*, 4*b*, 4-I, 4-II, 5, and 5-I all received once a week 3 to 4 drops of haliver oil with viosterol.

Strains of Parasites.—The strains of *P. lophurae* were all derived from strain 12A of Coggeshall (16). One strain has been maintained in baby chicks and passed by intracerebral inoculation every 6 days. It represents the original 12A strain except that it was passed once through *Aedes aegypti* mosquitoes before being returned to chicks and used for the present experiments. It was used for all the tests with chickens. Three sub-strains have been maintained in ducks. One (D-1) was derived from the original chick strain before mosquito passage and was used only for Experiment 1 of the experiments described in this paper. The second strain (D-2) was obtained from D-1 by infecting *Aedes albopictus* mosquitoes and then allowing them to feed on a duck. This strain, used in Experiments 2 and 11, has consistently been less virulent than D-1 and than most of the duck strains described in the literature. The third strain (D-3) was derived from the chick strain after mosquito passage and is about as virulent as strain D-1. It has been used for the biotin injection experiments. All the duck strains were passed by intravenous inoculation every 5 to 6 days.

Strain 3T of *Plasmodium cathemerium* in ducks was obtained through the kindness of Dr. Fruma Wolfson.

Inoculation of the Animals.—In each experiment the same infected blood was used

for the inoculation of all the animals. Chickens were infected with *P. lophurae* by injecting them in the neck vein with pooled heparinized blood from 1 week old chicks which had been infected 5 days previously. Ducks were infected *via* the neck or leg vein with heparinized blood, taken on the 5th day of infection, from a single donor duck 2 to 8 weeks old.

Biotin Assays.—The microbiological assay method of Shull, Hutchings, and Peterson (17) was used, with some minor modifications. The preparation of the basal medium was modified in such a way as to permit its use for the assay of pantothenic acid as well as biotin. The yeast extract was prepared by dissolving 20 gm. of Difco bacto-yeast extract in 200 ml. of 0.5 N sodium hydroxide and autoclaving for 30 minutes at 15 lbs., as in the pantothenic acid assay method (18). The solution was neutralized with glacial acetic acid, boiled, and filtered. The filtrate was diluted to 1 liter, brought to pH 2 with concentrated sulfuric acid, and treated with norit A and then with Superoxol in the manner described by Shull *et al.* (17). The adenine-guanine-cystine solution was prepared using the amounts given by Landy and Dicken (19). The stock solution mixture for biotin assay was as follows: H_2O_2-treated hydrolyzed casein solution, 200 ml.; alkali and H_2O_2-treated norit yeast filtrate, 200 ml.; vitamin solution (as described in (17) but exclusive of calcium pantothenate), 4 ml.; calcium pantothenate solution (20 mg. dissolved in 50 ml. water), 1 ml.; adenine-guanine-cystine solution, 200 ml.; tryptophane, 150 mg. plus asparagine, 400 mg., first dissolved in 50 ml. hot water; and water to make a total volume of 1 liter. For pantothenic acid assay the stock solution mixture was prepared in the same way except that the calcium pantothenate was omitted and biotin was added as 0.4 ml. of a solution of 25 γ of the free acid per ml.

Total biotin (20) was always determined. The sample of 0.5 ml. of blood, plasma, or red cells, or 0.5 gm. of minced liver, was autoclaved for 1 hour at 15 lbs. in 5 ml. of 3 N sulfuric acid. The material was filtered, the residue washed with a little distilled water, and the combined filtrates neutralized with 10 N sodium hydroxide and diluted to 15 ml. with distilled water. Such preparations from blood were assayed at concentrations of 0.4, 0.7, and 1.0 ml. per 10 ml. of culture medium. Preparations from liver had to be greatly diluted with water and were likewise assayed at three different levels. Material for pantothenate assay was autoclaved at neutrality in water and was tested at three different dilutions.

Lactobacillus casei ϵ (American Type Culture Collection No. 7469) was used throughout. It was maintained in culture following the method of Snell and Strong (21) and the inoculum was prepared after the manner of Landy and Dicken (19). For each series of biotin assays, a standard curve was prepared from the results with tubes containing known amounts of biotin ranging from 0.05 to 1.0 mγ per 10 ml. Growth was determined by titrating with 0.1 N sodium hydroxide the acid produced in the cultures after 3 days' incubation at 38°C.

In a recovery experiment, 2.5 mγ of pure biotin were added to each of six 0.5 ml. samples of chicken blood. These were then assayed for biotin together with corresponding 0.5 ml. samples of blood, to which no biotin had been added, from the same six chickens. The greatest error in recovered biotin was 25 per cent, and the average for the 6 determinations was 2.57 mγ biotin recovered.

The Effect of Egg White Diets on Susceptibility of Ducks and Chickens to P. lophurae

Charts 1 to 5 give the results of four typical experiments which illustrate the effect of biotin deficiency, induced by a high egg white diet, on the susceptibility of ducks and chickens to *P. lophurae*. It is apparent that, for both species of

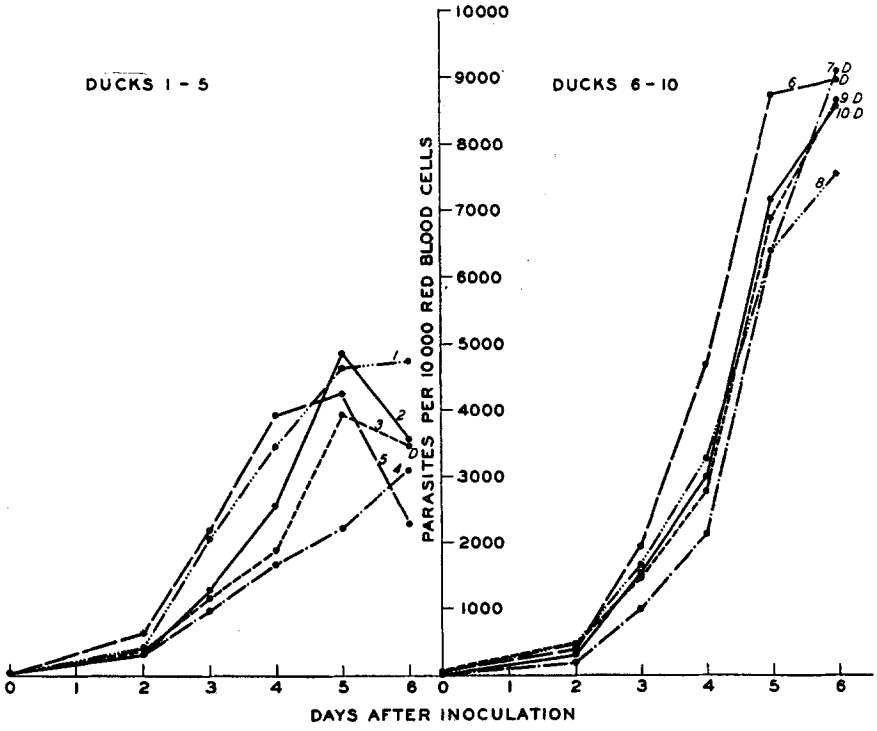

CHART 1. Experiment 1. Ducks 1 to 5 fed casein diet 1*a*; ducks 6 to 10, egg white diet 1. Inoculated with *P. lophurae* when 14 days old. Average weights when inoculated: 1 to 5, 105 gm.; 6 to 10, 120 gm. In this and succeeding charts, D signifies that animal died on day of last parasite count.

hosts, the average peak parasite number was 50 to 100 per cent higher in the biotin-deficient animals than in the controls, the highest peak was always reached in a deficient animal and the lowest in a control, and more of the deficient animals died of the infection. Appropriate uninfected control animals very rarely died of biotin deficiency alone when only 3 to 4 weeks old. It is noteworthy that in Experiments 1 to 4 the biotin-deficient and the control animals differed but little in weight. Indeed, in Experiments 1 to 3 the animals on egg white diet weighed a little more than the controls. It seemed probable

that the egg white supplied an unusually large amount of riboflavin, and accordingly in other experiments the control casein diets (as 3*b*, 4*b*) were supplemented with riboflavin at the rate of 5 mg. per 100 gm. of casein. When this was done, the animals on casein diet always grew more rapidly than those on egg white diet, but the responses to infection with *P. lophurae* were exactly the same as when additional riboflavin was not supplied.

When the young chickens had been on an egg white diet for about 2 weeks they began to show the syndrome first described by Ringrose, Norris, and

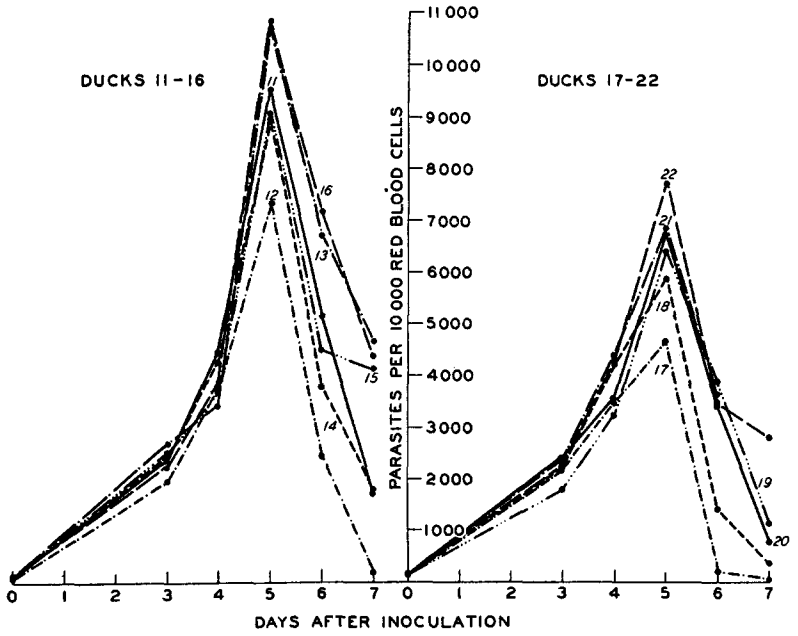

CHART 2. Experiment 2. All fed diet B for first 4 days. Thereafter ducks 11 to 16 were fed egg white diet 3, while ducks 17 to 22 were continued on diet B. Inoculated with *P. lophurae* when 13 days old. Average weights when inoculated: 11 to 16, 127 gm.; 17 to 22, 98 gm.

Heuser (22) and illustrated in Fig. 1. The down and feathers of the deficient chicks had a generally rough appearance, the feet showed a marked scaly dermatitis, and lesions appeared at the corners of the mouth and over the eyes. Some individuals developed perosis and in some the upper portion of the beak grew in a curved manner resulting in malocclusion. The animals on the various casein diets showed no signs of dermatitis. The early lesions of biotin deficiency in ducks are illustrated in Fig. 2. The down on the face, neck, and back of ducks on egg white diet presented a matted, rough appearance and sometimes fell, or was pulled, out, leaving bald areas. Lesions appeared around the eyes of the animals and their legs were frequently bowed. They were, however, just as

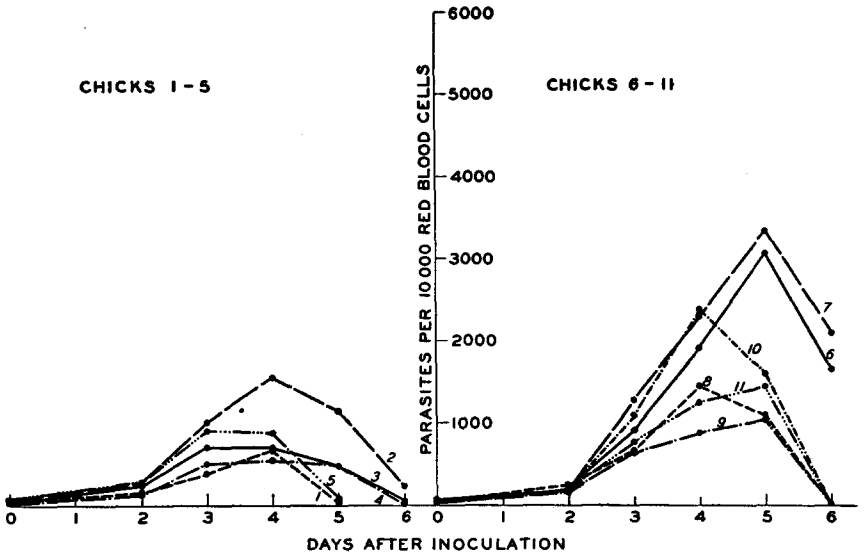

CHART 3. Experiment 3. All fed diet A for first 5 days. Thereafter chicks 1 to 5 were fed casein diet 2*a* and chicks 6 to 11, egg white diet 2. Inoculated with *P. lophurae* when 15 days old. Average weights when inoculated: 1 to 5, 92 gm.; 6 to 11, 98 gm.

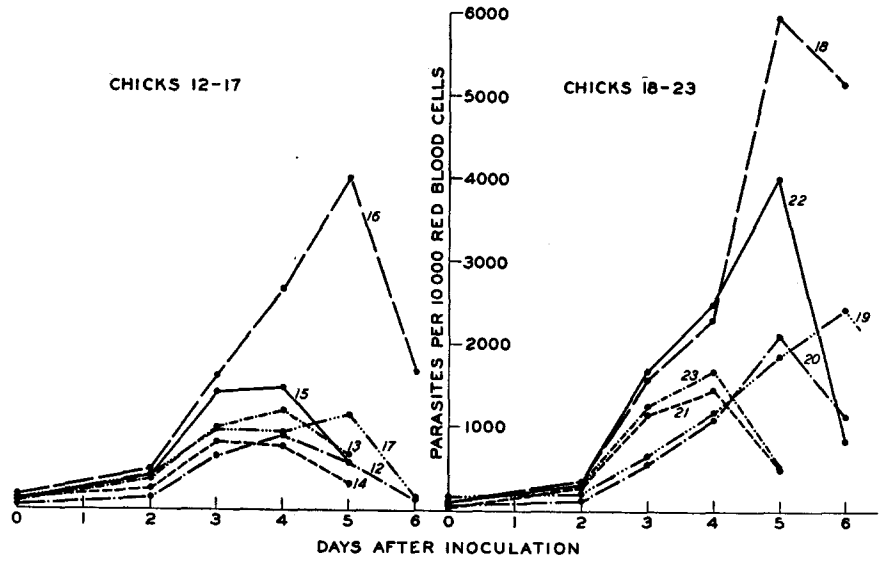

CHART 4. Experiment 4. Chicks 12 to 17 fed casein diet 2*a*, chicks 18 to 23 fed egg white diet 2. Inoculated with *P. lophurae* when 22 days old. Average weights when inoculated: 12 to 17, 133 gm.; 18 to 23, 105 gm.

active, when 3 to 4 weeks old, as the control animals. Ducks kept on a casein diet, such as 3*b* or 4*b*, eventually developed signs similar to those of ducks on egg white diet but much less marked. Rough down and bowing of the legs occasionally appeared even in ducks kept on diet B supplemented with lettuce, but all these abnormalities appeared regularly, early, and to a marked degree, only in ducks fed a high egg white diet.

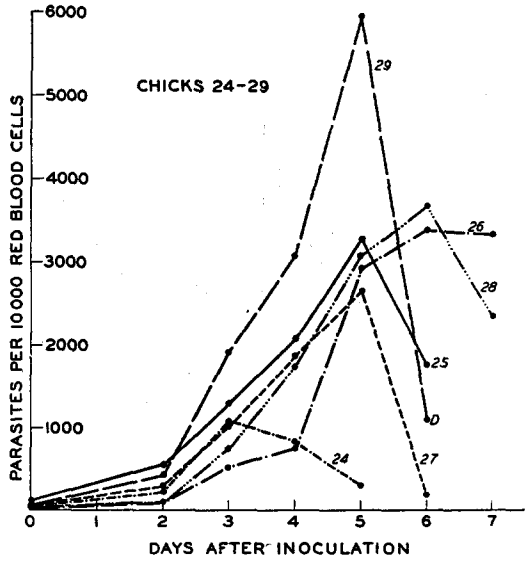

CHART 5. Experiment 4. Chicks 24 to 29 fed casein diet 2*a* for first 5 days, thereafter egg white diet 2. Inoculated with *P. lophurae* when 22 days old. Average weight when inoculated, 122 gm. For controls see Chart 4, chicks 12 to 17.

The Effect of Pantothenic Acid Deficiency and of Small Degrees of Biotin Deficiency

Since the biotin-deficient animals eventually die of the deficiency, it seemed possible that their greater susceptibility to *P. lophurae* might be merely the result of some general lowering of resistance, not necessarily specifically associated with the biotin level. It was therefore of interest to weaken the animals by means of some other nutritional deficiency. Pantothenic acid was selected since it is essential for the growth of chicks and since a deficiency of it produces lesions at the corners of the mouth and on the eyelids somewhat resembling those seen in biotin deficiency. Chickens maintained on the heated diet 5 showed the typical signs of pantothenate deficiency (Fig. 3) and were smaller and weaker than comparable chicks fed on egg white diet. The control chicks, which received diet 5-I, were vigorous and normal in every respect except for a slight scaliness on the feet. Ducks fed the pantothenic acid-de-

ficient diet (Fig. 4) grew very poorly and soon became weak and unable to open their eyes. Several died when they were only 2 weeks old and before they had been inoculated with malaria parasites. The control ducks fed diet 5-I appeared entirely normal. The average pantothenate content of the blood was, in mγ per ml., 167 for 2 deficient ducks and 293 for 3 ducks on the adequate diet. Yet in spite of the great difference in general health between the animals deficient in pantothenic acid and those not deficient in this vitamin, the former did not develop any more severe infections with *P. lophurae* than did the latter. The result obtained with the chicks (Chart 6) was especially instructive. It is at once apparent that, while the small, weak, pantothenic acid-deficient

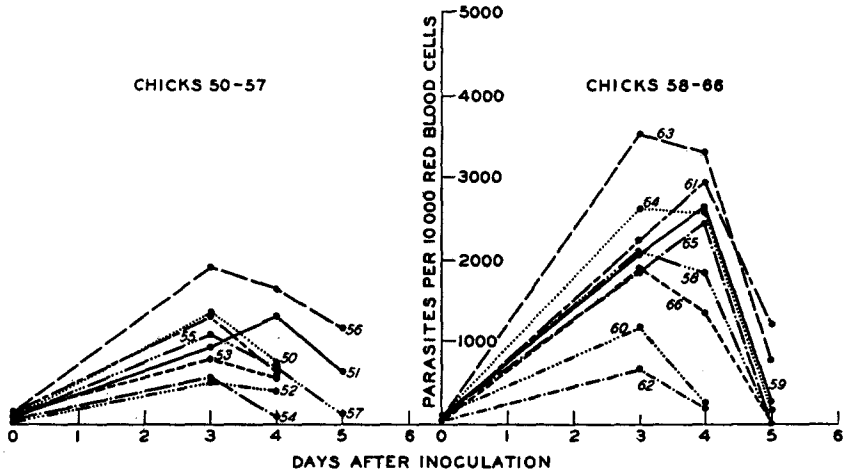

CHART 6. Experiment 5. Chicks 50 to 57 fed heated diet 5, deficient mainly in pantothenic acid; chicks 58 to 66 fed diet 5-I (diet 5 + 15 mg. calcium pantothenate per kg.). Inoculated with *P. lophurae* when 25 days old. Average weights when inoculated: 50 to 57, 96 gm.; 58 to 66, 133 gm.

chickens had mild infections, exactly of the type to be expected in chickens of their age, 5 out of 9 large, vigorous animals on the diet supplemented with pantothenate developed unexpectedly heavy infections and only 2 had very mild infections. As has already been stated, the chickens not deficient in pantothenic acid showed a scaly dermatitis of the feet, which is a characteristic sign of partial biotin deficiency. It has recently been shown (23) that a heated diet supplemented with thiamin, riboflavin, 2-methyl-1,4-naphthoquinone, and calcium pantothenate, such as diet 5-I, is still somewhat deficient in biotin. It has also been frequently observed (24, 23) that rapidly growing animals show the most distinct signs of biotin deficiency. It therefore seems likely that the pantothenic acid-deficient chicks, which failed to grow well, received enough biotin from the diet, while the chicks which received adequate pantothenic acid

and which grew rapidly were unable to obtain enough biotin. Their partial biotin deficiency would then account for their greater susceptibility to infection with *P. lophurae*.

Other types of experiments provide still better evidence for the effects of small degrees of biotin deficiency on the severity of *P. lophurae* infections.

In Experiment 6 (Chart 7) one group of chickens was kept on casein diet, one on egg white diet, and one on egg white diet supplemented with biotin (diet 4-I). The amount of biotin added in diet 4-I was calculated on the power of commercial egg white to inactivate biotin (25) and the average biotin content of chick feeds, and should have been enough to leave 20 γ per 100 gm. of diet. For about the first 10

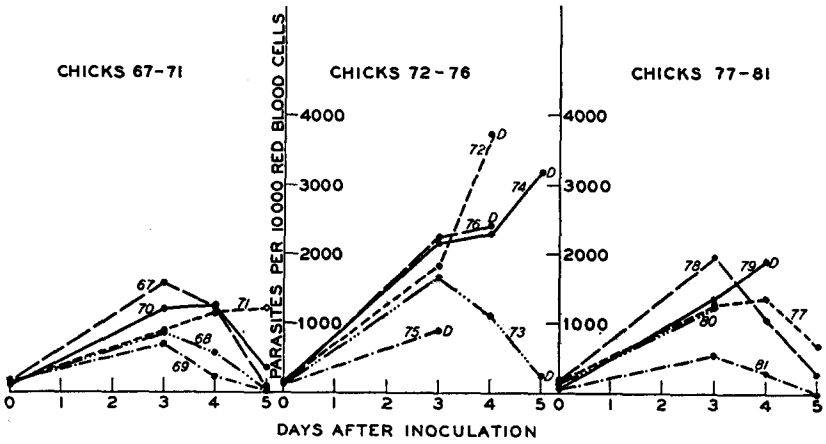

CHART 7. Experiment 6. Chicks 67 to 71 fed casein diet 4*a* for first 16 days, thereafter casein diet 4*b*; chicks 72 to 76 fed egg white diet 4; chicks 77 to 81 fed egg white + biotin concentrate diet 4-I for first 18 days, thereafter egg white + biotin concentrate diet 4-II. Inoculated with *P. lophurae* when 25 days old. Average weights when inoculated: 67 to 71, 176 gm.; 72 to 76, 122 gm.; 77 to 81, 149 gm.

days, the chicks fed diet 4-I showed even better growth than those fed the casein diet 4*a*. But by the time they were 2 weeks old they began to show signs of biotin deficiency which were almost as bad as those shown by the chicks on plain egg white diet 4. It was apparent that the biotin which had been added was inadequate. Woolley and Longsworth (26) had in the meantime reported almost twice as high a biotin-inactivating power for pure antibiotin (avidin) as had been reported by Eakin, Snell, and Williams (27). The amount of biotin concentrate added to diet 4 was therefore doubled when the chicks were 18 days old. Although they still showed signs of biotin deficiency when they were inoculated a week later with *P. lophurae*, they developed less severe infections than the chicks on unsupplemented egg white diet and slightly more severe infections than the chicks on casein diet.

In Experiment 7 (Table I) all 21 chicks were maintained from the day of hatching on a high egg white diet (diet 4). One group of 7 chicks received no other treatment.

These chicks all showed definite signs of biotin deficiency by the time they were inoculated with *P. lophurae*. The 7 chicks of the 2nd group received daily by intraperitoneal injection enough biotin concentrate (S.M.A. Co. No. 1000) diluted with 0.85 per cent salt solution to furnish 1 γ of biotin per chick. These chicks grew well and showed no signs of biotin deficiency other than a distinct, but not at all severe, scaly dermatitis of the feet. Each chick of the 3rd group received intraperitoneally enough biotin concentrate to furnish 3 γ daily for the first 17 days and 6 γ daily thereafter. Five of these 7 chickens showed no signs of biotin deficiency at the time they were inoculated, in agreement with the reported biotin requirement of chicks of about 3 to under 10 γ (28, 29). The other 2 had been exceptionally small, weak chicks since the date of hatching and they failed completely to respond to the biotin injections. When they were 20 days old, they were as small and showed as distinct

TABLE I

Experiment 7. The Effect of Intraperitoneal Injections of Biotin Concentrate on the Course of P. lophurae Infections in Chickens Fed Egg White Diet 4 and Infected When 20 Days Old

7 chickens in each group

Treatment	Average weight when inoculated	Parasites per 10,000 red cells				No. of chickens	
		Initial No.		Peak No.		Parasites cleared out by 5th day	Died
		Range	Average	Range	Average		
	gm.						
No biotin	114	120–250	177	3750–12,120	7400	0	3
1 γ biotin daily	131	135–215	181	3860–7480	5600	0	0
3 γ biotin daily for 17 days, then 6 γ biotin daily	150	160–295	214	2380–7920 (2380–5500)*	4900 (3900)*	2	0

* These values obtained if results with 2 obviously deficient chicks are omitted from this group.

signs of biotin deficiency as did the chicks in the group receiving no biotin. All the chicks were inoculated with a very large dose of *P. lophurae*. By far the highest peak parasite number occurred in the group receiving no biotin and the lowest in the group receiving the larger amount of biotin. The average peak parasite number was highest for the no biotin group, lowest for the high biotin group, and intermediate for the low biotin group. Especially interesting was the fact that the 2 chicks in the high biotin group which failed to respond to the biotin injections both had peak parasite numbers over 7,000. If the results with these 2 chicks are omitted, the range and average peak parasite numbers for the group become considerably lower. Only in the group receiving high biotin were there any chicks which had very few parasites by the 5th day (5 and 20 per 10,000 red cells). In the other two groups, and expecially in the group receiving no biotin, the parasite number was still up in the thousands on the 5th day. Deaths from the infection occurred only in the group receiving no biotin, where 3 out of the 7 chickens died 5, 6, and 8 days, respectively, after inoculation.

In this experiment the chickens receiving only 1 γ of biotin daily were active, well grown, and of normal appearance except for the mild scaliness of the feet. Yet they had more severe infections than the chickens receiving more nearly adequate amounts of biotin. It is evident that in the presence of a small degree of biotin deficiency the administration of biotin may be considered as a specific measure lessening the severity of the infection with *P. lophurae*.

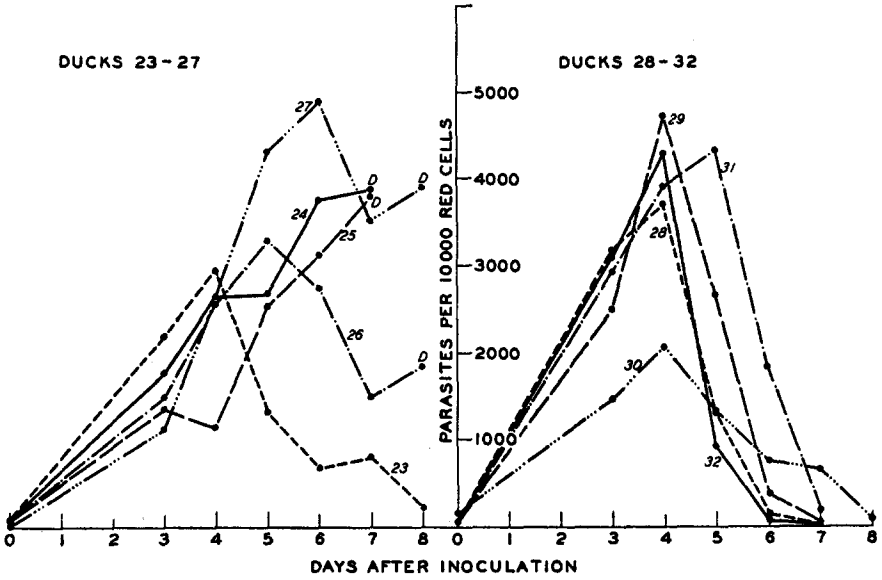

CHART 8. Experiment 8. Ducks 23 to 27 fed egg white diet 4, ducks 28 to 32 fed casein diet 4*b*. Inoculated with *P. cathemerium* strain 3T when 21 days old. Average weights when inoculated: 23 to 27, 200 gm.; 28 to 32, 349 gm. Average initial parasites per 10,000 red cells: 23 to 27, 79; 28 to 32, 91.

The Influence of Egg White Diets on Susceptibility to P. cathemerium

The effect of biotin deficiency on susceptibility has been studied with two species of bird malaria parasites other than *P. lophurae*. Experiments with *P. cathemerium* strain 3T gave especially interesting results, illustrated in Chart 8.

In the non-deficient ducks on the casein diet, the parasite count rose very rapidly for the first 4 days and then fell off equally abruptly. As is usual with *P. cathemerium* infections in ducks, none of the animals died (30). The infections in the biotin-deficient ducks did not attain any higher peaks than in the non-deficient ones. Indeed, the parasite count at first rose more slowly in the former than in the latter animals. But in only one (No. 23) of the deficient animals was the parasite peak

reached on the 4th day and followed by an abrupt decline in the number of parasites. In one duck (No. 26) the parasites increased to a peak on the 5th day, fell off somewhat on the 6th and 7th days, and rose again on the 8th day, when the animal died. In one (No. 27) the parasites increased to a high peak on the 6th day, fell off slightly on the 7th day, and rose again on the 8th day, when the animal died. In the other 2 ducks (Nos. 24 and 25) the parasites continued to increase until the 7th day, when both animals died. Control uninfected biotin-deficient animals survived 1 to 2 weeks or longer beyond the end of the experiment.

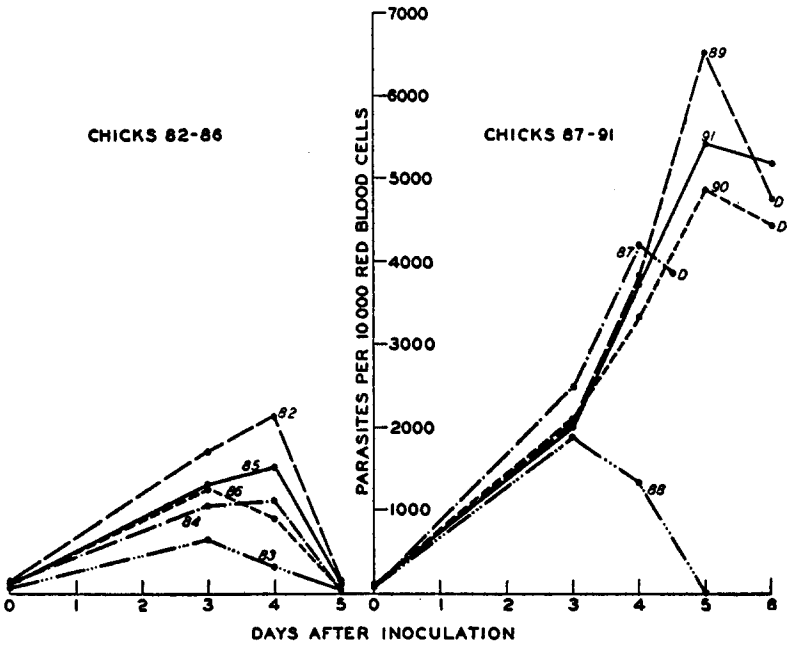

CHART 9. Experiment 9. Chicks 82 and 83 fed casein diet 4*a* for first 16 days, thereafter casein diet 4*b*; chicks 84 to 86 fed egg white + biotin concentrate diet 4-I for first 18 days, then egg white + biotin concentrate diet 4-II until 46 days old, thereafter casein diet 4*b*; chicks 87 to 91 fed egg white diet 4 throughout. Blood for biotin assays taken when 46 days old. Inoculated with *P. lophurae* when 48 days old. Average weights when inoculated: 82 to 86, 402 gm.; 87 to 91, 161 gm. Average initial parasites per 10,000 red cells: 82 to 86, 124; 87 to 91, 83. Average biotin, as mγ per ml. of blood: 82 to 86, 3.0, 87 to 91, 1.2.

Thus, in the non-deficient animals the parasites increased very rapidly at first, but were then equally rapidly removed from the circulation; while in the biotin-deficient ducks the parasites at first increased more slowly, but they continued to increase and could not be successfully cleared out of the blood stream.

The Biotin Content of the Blood in Relation to Infections with P. lophurae

The question arises as to what precisely are the biotin levels associated with different degrees of susceptibility. A small beginning toward answering this question has been made with the parasite *P. lophurae.* Although it was recognized that the level of biotin in the blood probably would not give an entirely accurate indication of the biotin level of the body as a whole, most of the biotin determinations were made on blood, since samples of this could be readily obtained without injuring the animal and since blood is the medium in which the malaria parasites live. Chart 9 illustrates the result of an experiment with chickens 48 days old when inoculated.

TABLE II

Experiment 10. The Relation between the Average Biotin Content of the Blood before Inoculation and the Average Peak Parasite Number for 3 Groups of 5 Ducks Each on 3 Different Diets, Inoculated with P. lophurae When 14 Days Old

Diet	Biotin mγ/ml. blood	Average weight when inoculated	Parasites per 10,000 red blood cells		Remarks
			Initial	Peak	
		gm.			
Egg white diet 4	1.8	167	116	8360	2 ducks still had high parasite counts (2940 and 6680) 11 days after inoculation and they died on the 13th day
Casein diet 4*a* for first 11 days, then 4*b*	3.0	118	115	6544	In both of these groups, the highest parasite count on the 11th day after inoculation was 115. Most of the ducks showed no parasites at this time and none died
Casein diet 3*a* for first 11 days, then 3*b*	3.0	149	110	6680	

Four of the 5 chickens in the group averaging before inoculation only 1.2 mγ of biotin per ml. of blood developed infections three times as severe as those developed by the 5 chickens with an average biotin of 3.0 mγ per ml. of blood. Three of the chickens with the low biotin died before the 7th day after inoculation and a fourth (No. 91) was killed on the 8th day, when it was very weak.

Table II illustrates again, for ducks, the inverse relation between blood biotin level and the average peak parasite number. Note the constancy of the results obtained with the two groups of ducks on two very different kinds of casein diets, which however had in common the property of not interfering with the biotin supply of the animals.

Since *P. lophurae* produces heavier infections, after the inoculation of comparable large doses, in ducks and in baby chicks than in older chickens, it was of

interest to compare the biotin levels in these 3 groups of animals. The biotin level of the blood of chickens kept on adequate diets did increase after they were 1 month old (Table III). However, the increase in the resistance of chickens to *P. lophurae* infections is quite notable by the age of 2 weeks, and at this time there appeared to be no higher biotin level either in the blood or in the

TABLE III

Changes with Age in the Biotin Content of the Blood of Individual Chickens on Egg White and Control Diets

Diet	Chick No.	Biotin, mγ/ml. blood at	
		31 days	46 days
Casein diet 4*a* for 14 days, then 4*b*	82	2.7	5.1
	83	2.8	4.6
Egg white diet 4 + 500 γ biotin per kg. for 16 days, then 1000 γ per kg.	84	1.2	2.3
	85	1.6	2.4
Egg white diet 4	87	1.8	1.2
	88	1.0	1.3

TABLE IV

The Biotin Content of the Blood and Liver of Young Ducks and Chickens

Age in days	Biotin per ml. blood		Biotin per gm. liver	
	Individual values	Average	Individual values	Average
	mγ	mγ	mγ	mγ
Ducks				
4	4.3, 4.0, 4.1	4.1	1260, 1540, 1720	1507
25	3.0, 3.2, 3.4	3.2	854, 1020, 1450	1108
67	2.3	2.3	1950, 1630	1790
88	4.9	4.9	2960	2960
Chicks				
2	2.6, 2.2, 2.2	2.3	2516, 2580, 3274	2790
14	3.0, 2.4, 3.2	2.9	2520, 1778, 1860	2053
26	2.5, 2.5, 2.3	2.4	2854, 1688, 3374	2639

liver than in 2 day old chicks (Table IV). Also, as shown in Table IV, the blood level of biotin is actually a little higher in ducks than in young chickens. The concentration of biotin in the liver of young ducks was found to be little more than half of that in the liver of young chicks, and it may well be that the general body level of biotin is higher in the latter than in the former.

In any case, the idea that a single static concentration of biotin in the blood determines the degree of susceptibility to *P. lophurae* infection is much too

simple to be expected to work. Rather one might expect that the important factors are the extent and nature of the reserve supply of biotin in the body, and the speed with which biotin can be mobilized into the blood stream. Some evidence has been obtained which shows that infection with *P. lophurae* modifies temporarily the biotin level of the blood of both chickens and ducks.

This was first observed in assays made with the chickens of Experiment 6 (see Chart 7). Besides the 5 chickens on each of the 3 diets which had been infected when 25 days old, several other chickens in each group were left uninfected (used later for Experiment 9). When the infected chickens were in the 6th day of their infection (except for chicks 73 and 80 which were bled on the 5th and 3rd day respectively),

TABLE V

The Biotin Content of the Blood of Uninfected 31 Day Old Chickens Kept on Egg White and Control Diets, and of Similar Chickens just Recovered from Infection with P. lophurae

Diet	Condition	Hemoglobin	Chick Nos.*	Biotin, per ml. blood	
				Range	Average
		per cent		*mγ*	*mγ*
Casein diet 4*a* for 14 days, then 4*b*	Not infected	85	82, 83	2.7–2.8	2.8
	Infected	55	67 to 71	4.0–6.9	4.9
Egg white diet 4	Not infected	85	87 to 91	1.0–2.0	1.6
	Infected	—	73		3.1
Egg white diet 4 + 500 γ biotin per kg. for 16 days, then 1000 γ per kg.	Not infected	85	84 to 86	1.2–2.0	1.7
	Infected	55	77, 78, 80, 81	3.1–4.4	3.6

* See also Experiments 6 and 9, Charts 7 and 9.

and both uninfected and infected ones were 31 days old, 1 ml. of blood was taken from each and assayed for biotin, with the results shown in Table V.

It is evident that, regardless of diet, animals just recovering from infection with *P. lophurae* and suffering from a considerable degree of anemia had about twice as much biotin per milliliter of blood as had uninfected animals. A similar result was obtained in assays on blood taken from ducks 6 days after inoculation with *P. lophurae*. Chart 10 shows the results of a more detailed analysis of this phenomenon carried out with 4 ducks on an adequate diet (diet B with lettuce)

A sample of blood was taken from each duck just before inoculation with *P. lophurae* and again on the 4th, 6th, and 8th days after inoculation. The blood was assayed for biotin. Blood films were made on the 4th, 5th, and 6th days so as to include the day of the peak parasite number. Both the plasma and red cell levels of biotin had

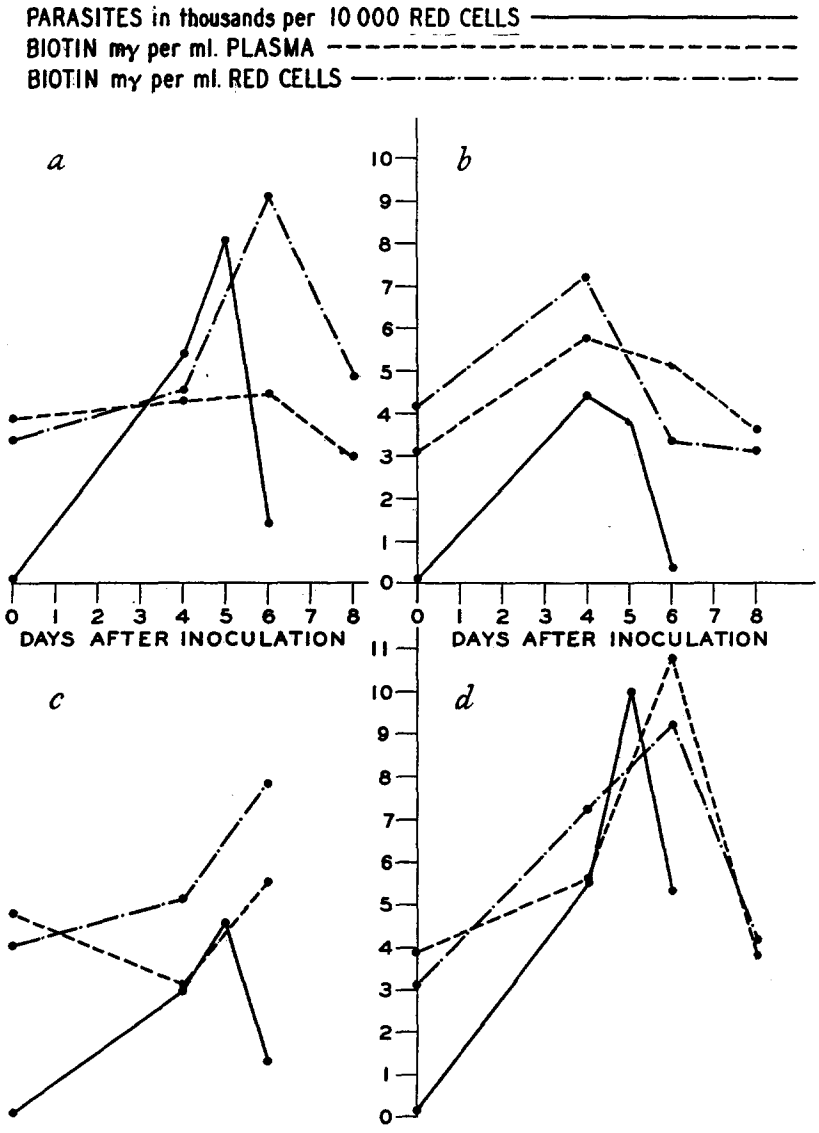

CHART 10. Experiment 11. Changes, during course of infection with *P. lophurae*, in the biotin content of the plasma and red blood cells of 4 ducks fed an adequate diet and inoculated when 16 days old.

already risen by the 4th day in all the ducks except duck *c*, whose plasma biotin fell while the red cell biotin rose. In duck *b*, whose infection reached its peak on the 4th day, the biotin in both plasma and red cells likewise reached a peak on the 4th day,

fell on the 6th day, and was back to its starting point on the 8th day, when very few parasites remained in the blood. In the other 3 ducks the peak parasite number occurred on the 5th day and the highest biotin levels on the 6th day. The biotin was again back to its original level by the 8th day (duck *c* died of the bleeding on the 6th day). Moreover, the extent of the increase in biotin level of both red blood cells and plasma was in general proportional to the extent of the parasitemia. This rise in biotin level cannot be explained solely on the basis of the new red cells formed in response to the anemia produced by the parasites (assuming that young red cells have a higher biotin content than mature ones). The increased biotin level was already apparent by the 4th day, when there was as yet no large proportion of young red cells; the increase appeared in the plasma as well as in the red cells; and both plasma and red cells were back to a normal biotin level by the 8th day after inoculation, when a large proportion of young red cells was still present.

These results suggest that biotin is mobilized into the blood stream during infection with *P. lophurae*, and that it may play a rôle in reducing the number of parasites.

A single, somewhat similar experiment (Experiment 12, Chart 11) has been performed with 4 chickens, 4 weeks old and weighing about 300 gm. at the start of the experiment.

Each of 2 of the chickens (*a*, *b*) received by stomach tube on 2 successive days a dose of 0.25 ml. of a solution of 0.2 gm. of phenylhydrazine hydrochloride in 10 ml. of water. At the same time that these chickens received their first dose of phenylhydrazine, the other 2 chickens were inoculated intravenously with *P. lophurae*. Blood for biotin assay was taken into a measured amount of heparin solution, by heart puncture, just before the first phenylhydrazine treatment or inoculation with parasites, and again 1½, 3, and 5 days later. This blood was centrifuged in a uniform way, the volume of red cells and plasma was noted, and measured amounts of plasma and red cells were assayed for biotin. Stained blood films were prepared daily and the relative numbers of parasites and young red cells were determined. The results are summarized in Chart 11.

It is apparent that the plasma biotin rose in chickens *c* and *d* just as it did in the ducks of Experiment 11. Especially notworthy is the fact that the plasma biotin in both chickens had doubled only 1½ days after inoculation. Thus, in chickens the biotin in the plasma not only attained higher levels than in the ducks in Experiment 11, but it did so much more quickly. If additional results of this type can be obtained, they will support the idea that older chickens infected with *P. lophurae* can clear out their infections more rapidly than ducks, partly because they can raise their plasma biotin more quickly and to a greater extent. The data for chickens *c* and *d* also show that the changes in plasma biotin are not connected with the anemia which follows the malarial infection. The level of biotin in the plasma was much increased 1½ days after inoculation, when there was no anemia, as evidenced by the normal volume per cent of red

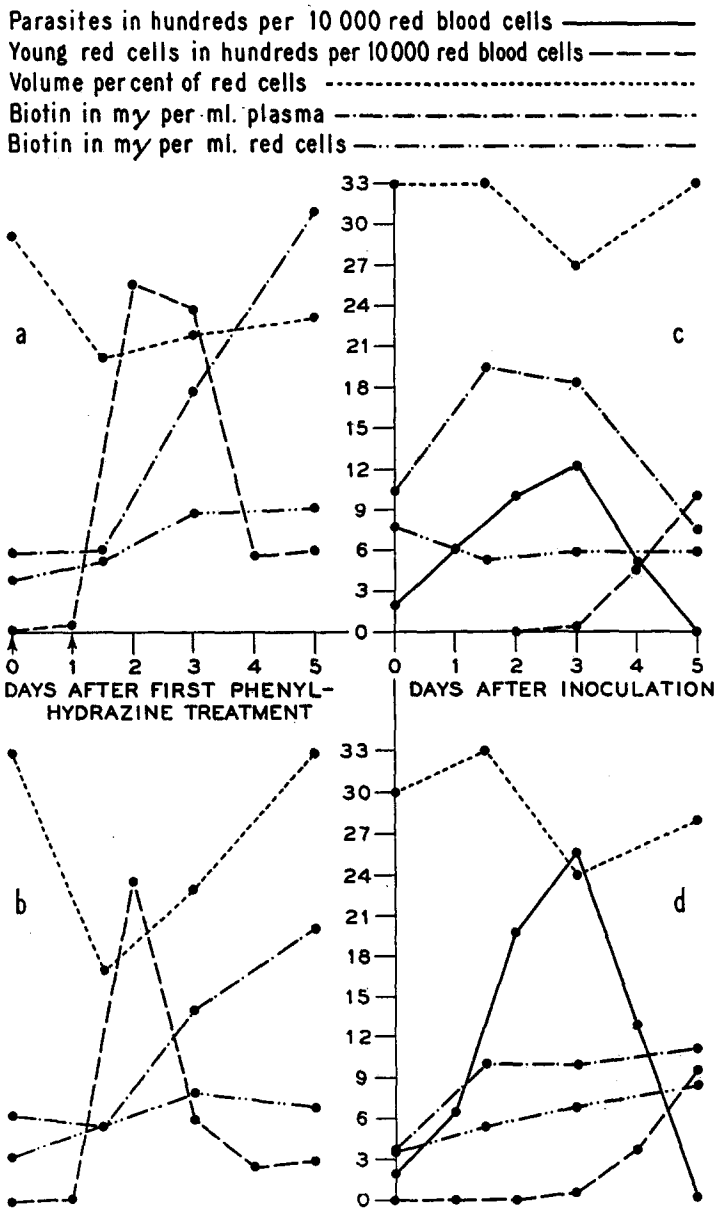

CHART 11. Experiment 12. Changes, during infection with *P. lophurae* and following phenylhydrazine treatment, in the erythrocytes and in the biotin content of erythrocytes and plasma of 4 week old chickens. Chickens *a* and *b* received phenylhydrazine on the days indicated by the arrows, while chickens *c* and *d* were inoculated intravenously with a large dose of *P. lophurae*.

cells (33 per cent) and the lack of young red cells. In chickens *a* and *b* young red cells were beginning to appear within 1 day after the first phenylhydrazine treatment and there was a marked anemia ½ day still later. The level of biotin in the plasma showed no increase at the time, but it was greatly increased on the 3rd day after the first phenylhydrazine treatment and continued to increase to the 5th day. Although the increase followed the production of large numbers of young red cells, it continued after the young red cell production had ceased. The young red cells themselves did not appear to have a sufficiently higher biotin content than the older ones (as evidenced by the relatively small increase in the biotin level of the red cells) to account for the great changes in the biotin level of the plasma. It also does not appear likely that the changes in plasma biotin can be explained directly on the basis of red cell destruction, since the red cells originally contain no higher concentration of biotin than the plasma. These results with phenylhydrazine suggest a method for artificially changing the plasma biotin level. It is very noteworthy that chickens treated with phenylhydrazine have been observed to develop less severe infections with *P. lophurae* than untreated chickens (31). This fact has been ascribed to the apparent preference of *P. lophurae* for mature erythrocytes, but the effect of phenylhydrazine in increasing the plasma biotin level may also play a part. Canaries treated with phenylhydrazine have been found to develop more severe infections with *P. cathemerium* than untreated birds, and this again has been ascribed to the preference of this parasite for young erythrocytes (32, 33). It has already been shown that the initial rate of multiplication of *P. cathemerium* is higher in ducks with a normal biotin level than in biotin-deficient ducks, a fact which fits very well with the idea that the effect of phenylhydrazine is partly a result of its action on the biotin level. Indeed, since the indications are that immature red cells do have a somewhat higher biotin content than mature ones, the so called preference of different species of malaria parasites for young or old red cells might be intimately connected with the relative biotin content of these cells.

Experiments on the Injection of Biotin into Ducks Kept on an Adequate Diet and Infected with P. lophurae

A number of preliminary experiments, four with S.M.A. biotin concentrate No. 1000 and one with pure biotin (very generously supplied by Dr. du Vigneaud) have failed to give consistent results.

In 3 of the experiments there seemed to be a small but significant decrease in the severity of the infections in the ducks injected with biotin as compared with the untreated ducks. In the other 2 experiments there was no effect of the biotin treatment. In one of these experiments biotin assays were made on the blood of treated and untreated ducks, 1 day after inoculation with *P. lophurae* and 12 hours after

the last intravenous injection of biotin into the treated animals (S.M.A. biotin concentrate No. 1000 to supply 25 γ of biotin per 100 gm. body weight).

In these assays there was actually more biotin in the blood of the untreated than in the blood of the treated ducks. West and Woglom (34) similarly found that excess biotin injected into mice was very rapidly excreted. Before a conclusive test can be made of the possible effects of a higher than normal blood level of biotin on infection with *P. lophurae*, it will be necessary to find a way of artificially maintaining over a period of several days a continuously high level of biotin in the blood.

DISCUSSION

Biotin is evidently a substance which affects the degree of natural susceptibility to malarial infection. How the biotin level of the blood exerts its influence upon the extent of multiplication of the parasite can at present only be guessed. A very simple theory would assume that all species of malaria parasites require biotin for growth (since highly specialized parasites probably cannot themselves synthesize biotin), that for each species of bird malaria parasite there is an optimum range of biotin concentration in the blood of the host, that growth of the parasites is slowed up if the biotin concentration falls below this range, and that growth is inhibited by a direct toxic effect of biotin concentrations above the range. On the basis of such a theory, *Plasmodium lophurae* would require a relatively low optimal concentration of biotin. As the infection progresses it causes the biotin of the blood to increase. When the biotin level exceeds the favorable concentration range, the multiplication of the parasite is greatly reduced and the acute infection is terminated. Biotin-deficient animals are less capable of increasing sufficiently their blood biotin level, and so more of them cannot bring their infections under control and more die from it than among the non-deficient animals. Perhaps chickens, which have a higher biotin content in the liver than ducks, can increase their blood biotin level more rapidly and effectively than ducks, accounting for their greater resistance. *P. cathemerium* must be assumed to require a higher but narrower optimal range of blood biotin concentration than *P. lophurae*, since this parasite multiplies very rapidly at first in normal ducks. If this infection also increases the blood biotin level, then the abrupt decline in normal ducks can be explained partly on the basis of too high a concentration of biotin. In the biotin-deficient ducks, *P. cathemerium* at first multiplies more slowly than in normal ducks, presumably because the blood biotin level is below the optimal range. But as the biotin concentration increases in response to the infection, it enters the optimal range and the parasites multiply more rapidly. The deficient ducks then have difficulty in raising the biotin concentration above the optimal range and most of them die with a high parasite number in the blood. It is obvious that while

this simple hypothesis could explain the facts thus far available, much more information is needed before it can be taken very seriously.

It is possible that biotin deficiency exerts some specific effects on cell systems, such as the lymphoid-macrophage system, which are intimately concerned with defense against malaria parasites (35), or that it interferes with protein metabolism and in this way with antibody formation (36). Either of these suppositions would encounter difficulties in explaining the initial slower multiplication of *P. cathemerium* in the deficient animals. Moreover, the evidence at present available indicates that the biotin level of the host affects the natural rather than the acquired resistance. Attempts to produce relapse, by means of biotin deficiency, in animals recovered from infection with *P. lophurae* failed. Such experiments should, however, be repeated with a species of parasite (as *P. gallinaceum*) which has a fairly high relapse rate.

Any attempt to explain the effect of biotin on susceptibility to malaria must also recognize the fact that there are undoubtedly many other substances within the host the concentration of which also affects the susceptibility. Some of these substances may well have a more striking effect than biotin, and the effect of the sum total of all such substances must determine the degree of susceptibility of a host to a parasite.

The relation of nutrition to resistance to disease is still far from clear. There can be no doubt that resistance to disease is greatly decreased in the presence of extensive nutritional deficiency, but under such conditions the complicating factors (such as inanition) are numerous and it is impossible to conclude that any specific relationship exists between a particular dietary factor and the degree of resistance to a particular infectious agent. However, when a mild degree of a specific nutritional deficiency, unaccompanied by any general severe weakening of the animal, is accompanied by changed susceptibility to an infectious disease, one may be justified in assuming that a specialized kind of relationship exists between the level in the host of the nutritional factor concerned and the susceptibility of the host to invasion by the parasite. Further justification for such an assumption is provided if, as in the present work, even extreme deficiency of some other nutritional factor does not lead to changed susceptibility. There are in the literature relatively few examples of such specific relationships between the level in the host of a vitamin and the degree of susceptibility to an infectious agent. Although Ackert, McIlvaine, and Crawford (37) in their original work on the effect of vitamin A deficiency in increasing susceptibility to helminth infections, failed to detect any effects of a subclinical degree of vitamin A deficiency, McCoy (38) found that rats depleted of vitamin A showed lowered resistance to *Trichinella spiralis* before there were any other signs of avitaminosis. Boynton and Bradford (39) had similarly found that the lowered resistance of rats on a vitamin A deficient diet to infection with a bacillus of the *Mucosus capsulatus* group was apparent before any

other signs of vitamin deficiency. The resistance of rats to infection with murine typhus rickettsiae was greatly lowered even by a mild degree of riboflavin deficiency, but was not affected by extreme vitamin A deficiency (40). Rats deficient in thiamin were more susceptible to rat leprosy (41) and mice partially deficient in thiamin or riboflavin were more susceptible to pneumococcal infection than animals receiving adequate amounts of these vitamins (42).

Of a somewhat different type are the results of Becker and his associates (43–45) on the effects of dietary factors on the extent of multiplication of the coccidial parasite of rats, *Eimeria nieschulzi*. If rats were kept on a diet low in thiamin and pyridoxine, the addition of thiamin decreased the severity of infection and the further addition of pyridoxine decreased it still more. However, if only pyridoxine was added, the infection was more severe than on the basal diet. In rats fed a diet partially deficient in pantothenic acid, the infection was less severe than in rats fed the same diet supplemented with calcium pantothenate. It has similarly been found that the mortality rate from poliomyelitis is higher in normal rats than in rats on a riboflavin-deficient diet (46), and that it is higher in rats on a high thiamin diet than in rats on a low thiamin diet (47). All these results, together with those reported in this paper, would certainly support the idea that the vitamins are among the substances whose concentration in a host animal influences the extent of growth or multiplication of certain parasitic agents. This influence could be just as direct as the influence on a free living protozoon of the concentration of growth substances in its environment.

Since individual animals are known to differ in their body level of growth factors, even though they are not deficient and are within the normal range, it may be that such differences help to account for individual differences in susceptibility.

SUMMARY

Biotin-deficient chickens and ducks developed much more severe infections with *Plasmodium lophurae* than did non-deficient control animals. While a very mild degree of biotin deficiency sufficed to increase susceptibility, even an extreme degree of pantothenic acid deficiency had no effect. Biotin deficiency also increased the susceptibility of ducks to *P. cathemerium*. In animals infected with *P. lophurae*, the concentration of biotin in the plasma as well as in the red cells rose during the course of the infection, reached a peak at about the same time as the parasite number reached its peak, and then returned to normal as the infection subsided. While the administration of additional biotin to animals partially deficient in biotin could be considered a specific measure tending to lessen the severity of infection with *P. lophurae*, the injection of biotin into animals fed a diet adequate in this vitamin had no antimalarial effects, perhaps because the excess biotin was rapidly removed from the blood.

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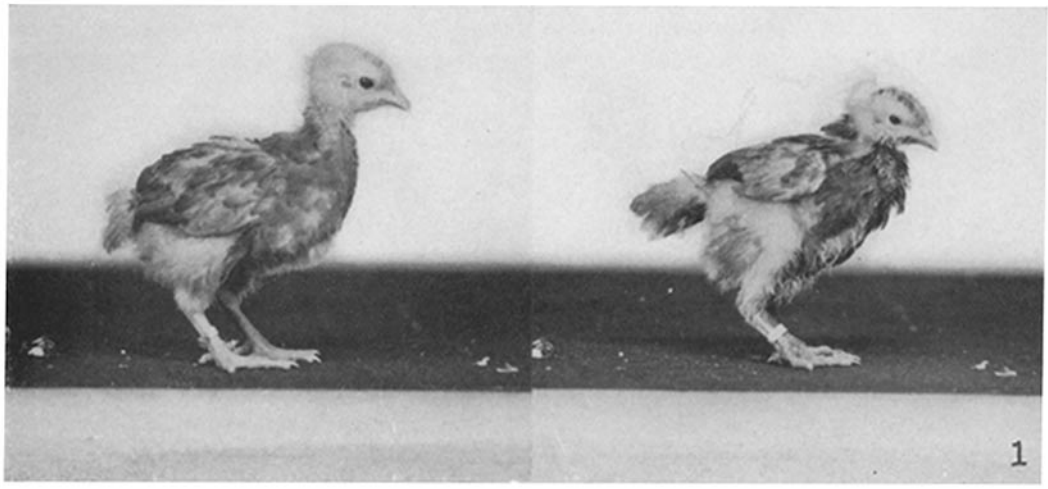
EXPLANATION OF PLATES

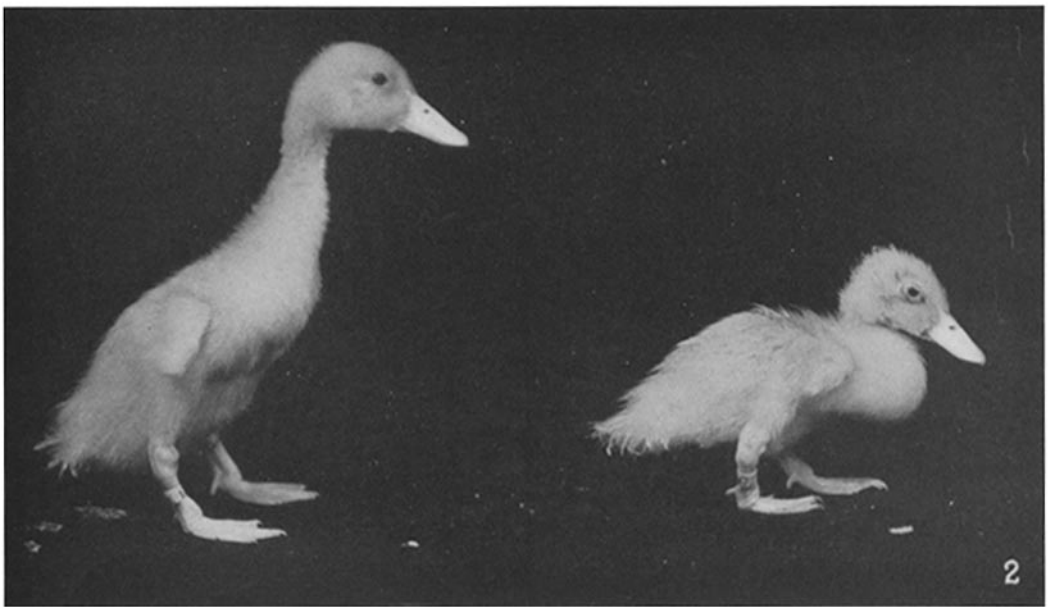
The photographs were made by Mr. J. A. Carlile.

PLATE 22

FIG. 1. Left: Chick fed casein diet 2*a*. Right: Chick fed egg white diet 2. Both 29 days old.

FIG. 2. Left: Duck fed casein diet 4*a* until 11 days old, thereafter casein diet 4*b*. Right: Duck fed egg white diet 4. Both 20 days old.



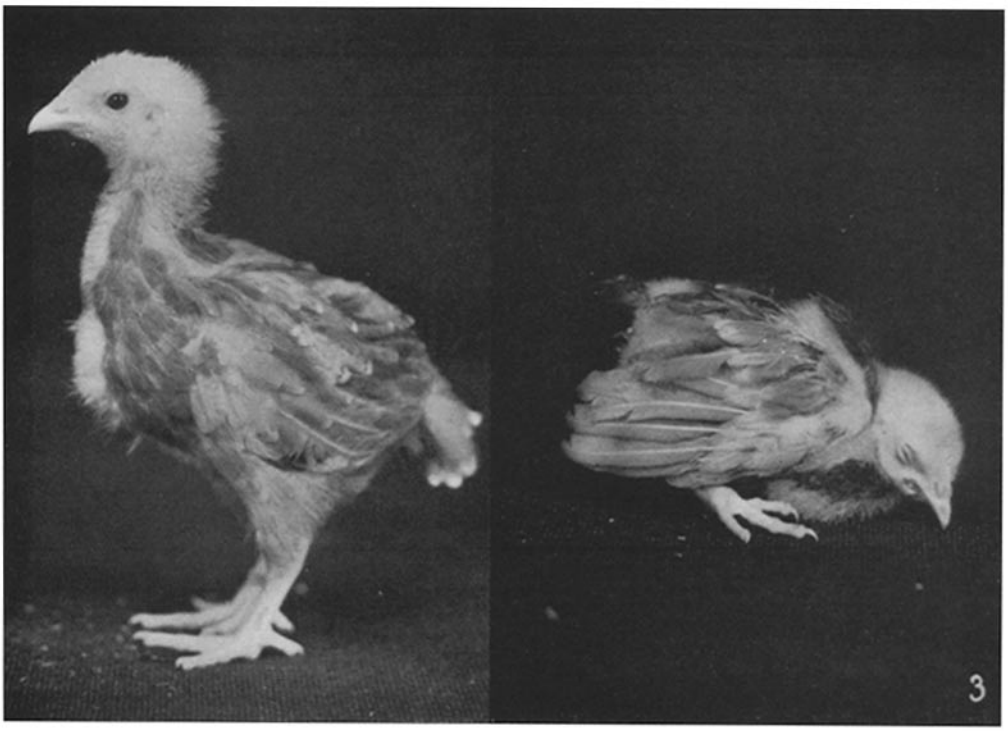


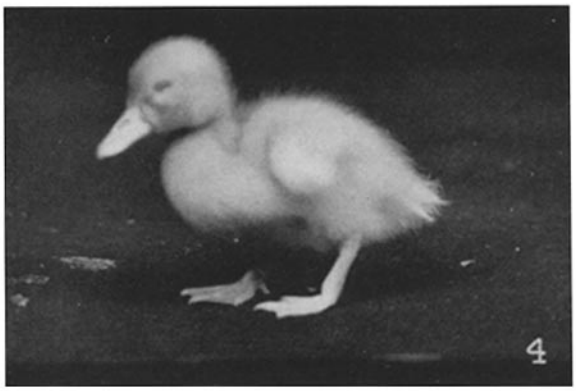
(Trager: Influence of biotin upon susceptibility to malaria)

PLATE 23

FIG. 3. Left: Chick fed diet 5-I adequate in pantothenic acid. Right: Chick fed diet 5 deficient in pantothenic acid. Both 33 days old.

FIG. 4. Duck fed diet 5 deficient in pantothenic acid. 13 days old.





(Trager: Influence of biotin upon susceptibility to malaria)