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THE RELATION TO THE COURSE OF AVIAN MALARIA OF BIOTIN AND A FAT-SOLUBLE MATERIAL HAVING THE BIOLOGICAL ACTIVITIES OF BIOTIN

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It has been shown that biotin deficiency decreases the resistance of chickens and ducks to avian malaria parasites (1, 2), of rats to the flagellate *Trypanosoma lewisi* (3), and of mice to the bacteria of mouse typhoid (4). The deficiency appears to interfere with the normal functioning of some general mechanism of resistance to infection. In the studies with avian malaria it was found that the concentration of biotin in the blood of infected animals increased during the period of parasite rise in number and decreased as the infection subsided (1). More extensive experiments have now confirmed the fact that the concentration of biotin in the plasma of chickens and ducks infected with malaria parasites undergoes characteristic changes. In addition, it has been found that the plasma of various species of animals contains a substance, probably a lipoprotein, which must be in some way related to the metabolism of biotin. This substance yields, after hydrolysis with acid or enzymes, a fat-soluble material having the biological activities of biotin but differing from it chemically. The nature and properties of this material, designated as FSF, have been considered in a separate publication (5). In the work reported in the present paper the biotin-like growth activity of FSF has been used as a method of estimating the relative concentration of the presumed lipoprotein from which it is derived. The changes in concentration undergone by this substance in the plasma of birds infected with malaria, as well as the antimalarial effects *in vitro* of plasma fractions relatively rich in it, suggest that it is more directly concerned with resistance to infection than is biotin itself.

Methods

The methods used have been much the same as those already described (1). The experimental animals were again young Rhode Island Red chickens and White Pekin ducks, both received as day old animals and reared in wire bottom cages until ready for use. The infections studied were *Plasmodium lophurae* (strain 12A) in both chickens and ducks and *P. cathemerium* (strain 3T) in ducks. Both species of parasites were obtained from Dr. A. P. Richardson of the Squibb Institute and represent different substrains from those used for the previous work. They were maintained by blood passage in ducks, *P. lophurae* being subinoculated once a week and *P. cathemerium* every 3 to 4 days. When *P. lophurae* was desired for an experiment in chickens, it was submitted to at least 3 passages at 5 to 6 day intervals in

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week old chicks before being used for the inoculation of the experimental chickens. All inoculations were intravenous. In some experiments relatively small numbers of parasites were given in order to prolong the course of the infection. Blood for subinoculation and for assay for biotin and FSF was taken from the neck vein of ducks and larger chickens, and from the heart of very young chicks.

Diets.—I. A chick-starting mash. Yellow corn meal 25.2, ground wheat 25.2, alfalfa leaf meal 6.5, soy bean meal 15, meat scrap 3.8, pinhead oats 13 (later omitted without any noticeable effect), cod liver oil 0.6, charcoal 0.6, salt 0.6, calcite 1.8, fine grit 3.8, fine oyster shell 3.8. The diet was used for both chickens and ducks and appeared to be quite adequate. It contained 12 to 13 γ of biotin per 100 gm. of diet.

II*a*. High egg white diet. 20 parts of commercial dried egg albumin mixed with 80 parts of diet I. This had a biotin content of 20 γ per 100 gm., but, due to the inactivation of the biotin by the excess of avidin (6), animals which were placed on it when 5 to 7 days old showed signs of biotin deficiency 2 weeks later.

II*b*. Control casein diet. 20 parts of a commercial washed casein, first mixed with riboflavin to provide 5 mg. riboflavin per 100 gm. casein, plus 80 parts of diet I. This had a biotin content of 13 γ per 100 gm. Animals fed diet II*a* or II*b* each received 3 drops of haliver oil with viosterol once a week.

III. Synthetic biotin-deficient diet. This was based on diets used in studies on chick nutrition (7, 8) and had the following composition per 100 gm.: in grams, white dextrin 51.5, vitamin-free casein 25, gelatin 10, salts V (7) 6, *l*-cystine 0.3, cellulose (Celluflour) 3, soy bean oil 4, choline chloride 0.2, Wilson's liver-fraction-L 2; plus, in milligrams, thiamin 0.4, riboflavin 0.8, pyridoxine 0.6, *d*-calcium pantothenate 1.5, niacin 3.0, *i*-inositol 50.0, para-aminobenzoic acid 15.0, 2-methyl-1,4 naphthoquinone 0.5, α-tocopherol 5.0. Each chick received 3 drops of haliver oil with viosterol once a week. This diet contained only 0.6 γ of biotin per 100 gm., and chicks placed on the diet when 2 days old showed signs of biotin deficiency in 2 to 3 weeks.

Assays for Biotin and FSF.—The acidimetric microbiological assay method employing *Lactobacillus casei* ϵ (9) has been used throughout. The medium and method of inoculation were those of Landy and Dicken (10) with slight modifications (5).

It has been found (5) that the normal plasma of several species of animals contains a low concentration of free biotin and no bound biotin whatever, since plasma hydrolyzed by acid or enzyme treatment and then filtered has the same biotin content as plasma merely diluted in water. However, unfiltered hydrolyzed plasma has a 4- or 5-fold higher biotin activity. This is due to the liberation by hydrolysis of the fat-soluble material FSF, which is readily removable by filtration or by shaking with ether and which has the activities of biotin. When this material was added at different appropriate concentrations to the basal medium used for biotin assay, the growth response curve given by *L. casei* had a shape very similar to that obtained with biotin itself (5). This fact has made it possible to measure with considerable accuracy the relative concentration of FSF in terms of its biotin activity. Since plasma contains no bound biotin proper, the difference in biotin activity between plasma merely diluted in water and plasma hydrolyzed by acid or enzyme treatment and not filtered must represent the relative concentration of bound FSF. Unhydrolyzed plasma contains a low and rather variable proportion of biotin activity which can be removed by shaking with ether. This may represent free FSF. Since its concentration was always low and underwent little change during the malarial infections it has been lumped together with the free biotin.

Thus, for most of the work, 2 measurements were made on each plasma. One, using whole plasma diluted in water, represented the free biotin, plus the small amount of free FSF. The other, using hydrolyzed plasma, represented the total biotin activity. The difference between the two represented the bound FSF. The details of the technique have been as follows. A

suitable quantity of blood, usually 2 ml., was drawn aseptically and placed in a graduated centrifuge tube containing a solution of heparin (27 mg. per cent) in 0.85 per cent sodium chloride solution, 0.15 ml. of heparin solution being used for each 2 ml. of blood. If the animal was infected, a drop of the same blood was used for the preparation of a blood film stained with Giemsa. The blood sample was centrifuged and the volumes of cells and plasma noted. The plasma was drawn off and 0.4 ml. of it was added to 11.6 ml. of sterile distilled water and thoroughly mixed. This gave the sample for the determination of free biotin. It was usually added to the assay tubes in amounts of 0.4, 0.8, and 1.3 ml. per 10 ml. of final medium. Another 0.4 ml. sample of the plasma was added to 4 ml. of 3 N sulfuric acid. This material was autoclaved at 15 pounds for 1 hour and then brought to a pH of about 8 with 10 N sodium potassium hydroxide and suitable small additions of 0.1 N sodium hydroxide or hydrochloric acid. It was finally diluted with water to 12 ml. to form an evenly turbid suspension. Upon standing, material of an obviously fatty nature gathered at the surface but this could be readily resuspended to again give an evenly turbid mixture. This preparation was used for the determination of total biotin activity. It was usually added to the assay tubes in amounts of 0.1, 0.2, and 0.4 ml. per 10 ml. of final medium. Both the plasma diluted in water and that added to the sulfuric acid could be stored at least a week in the refrigerator without any change in their biotin activity.

Organs, *e.g.* liver, were prepared for assay for their biotin content by autoclaving for 1 hour at 15 pounds a weighed, minced sample in 3 N sulfuric or 6 N hydrochloric acid, followed by filtration and neutralization of the filtrate. The FSF content of organs was also determined in a few experiments by neutralizing the acid-autoclaved suspension, extracting an aliquot of it with ether, and comparing the biotin activity before and after ether extraction, the difference being considered as activity due to FSF.

Judging from the recoveries obtained when known amounts of pure biotin were added to plasma, from the results with the 3 different concentrations of each sample, and from the general consistency of the results, the biotin and FSF assays were accurate to within 10 to 15 per cent.

The Effect of a Synthetic Biotin-Deficient Diet

Since in the earlier work (1, 2) biotin deficiency was produced by a diet high in egg white, the possibility existed that egg white itself might be exerting some effect aside from the production of the deficiency. This possibility has been ruled out by two experiments with chickens fed the synthetic biotin-deficient diet III and inoculated with *P. lophurae* when 3 weeks old. When chickens on this diet received by dropper 10 γ of biotin twice weekly they developed less severe infections with *P. lophurae* than did the unsupplemented chickens. The results of one experiment are illustrated in Fig. 1. The average peak parasite numbers were 9524 per 10,000 red cells for the biotin-deficient chicks and 5376 for the chicks receiving the biotin supplement. A third group of chicks included in this experiment and fed a natural diet (I) had an average peak parasite number of only 3550, representing perhaps the effect of a more nearly adequate intake of folic acid (11) or of some other unknown dietary factor. The average parasite counts on the 4th day after inoculation were 4276 for the biotin-deficient group, 2972 for the supplemented group, and 2020 for the group on diet I.

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The Effect of Different Degrees of Biotin Deficiency on Plasmodium cathemerium Infection in Ducks

In the previous work (1) it was reported that *P. cathemerium* at first multiplied more slowly in biotin-deficient ducks than in control animals. Later the infections in the deficient ducks overtook those in the controls and reached higher peaks. It has now been found that the original relative inhibition of multiplication is greater the more severe the biotin deficiency at the time of inoculation. This fact is illustrated in Table I. The effect is similar to that

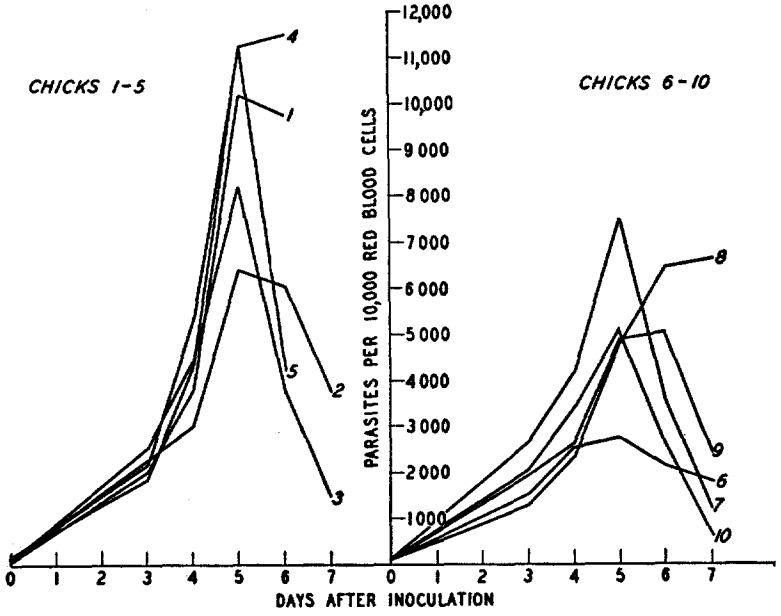

FIG. 1. The course of *P. lophurae* infection in chicks fed a synthetic biotin-deficient diet (chicks 1 to 5) and in chicks fed the same diet plus a biotin supplement (chicks 6 to 10). The chickens were inoculated when they were 23 days old.

obtained with riboflavin deficiency and *P. lophurae* in chickens (12), and with pantothenate deficiency and *P. gallinaceum* also in chickens (13). It is presumably a result of a direct effect on the parasite of too low a level of the growth factor, and resembles the effect of pantothenate deficiency on coccidia first reported by Becker and Smith (14). It emphasizes the fact that different degrees of the same deficiency may have two opposite effects, one to interfere with some defense mechanism of the host, the other to inhibit the growth of the parasite. In biotin deficiency the first effect appears with a relatively mild deficiency and is by far the more conspicuous one. The second effect has not been seen at all with *P. lophurae*. Even with *P. cathemerium* it is prominent only when the deficiency is severe and other complicating factors may be entering in.

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The Lack of Effect of Biotin Deficiency on the Leucocytes of Chickens

Six chickens made biotin-deficient on diet II*a* had total leucocyte counts at the age of 21 days ranging from 11,000 to 34,000 and averaging 23,000 per c. mm. Six control chickens of the same age maintained on diet II*b* had counts ranging from 16,000 to 24,000 and averaging 21,500. All 12 chickens were inoculated with *P. lophurae* when they were 23 days old. Four days later the leucocyte counts were: on diet II*a*, 12,000 to 44,000, average 30,000; on diet IIb, 25,000 to 51,000, average 34,100. The average peak number of parasites was 7050 for the group on diet II*a* and only 3250 for the group on diet II*b*.

TABLE I

*The Effect of Biotin Deficiency on the Course of Plasmodium cathemerium Infection in Ducks**

Experiment No.	Diet	No. ducks	Degree of deficiency	Average parasites per 10,000 red cells on days after infection				Deaths
				0	2	3	4	
1	II*a* egg white	8	Moderate	100	1460	5380	6525	7 on days 4–7
	II*b* casein	8	None	70	1640	4170	4625	0
2	II*a* egg white	3	Severe	80	730	—	—	3 on days 2–4
	II*b* casein	4	None	80	1560	4800	4610	0

* The ducks of Experiment 1 were kept 5 days on diet I and 19 days on the experimental diets before being inoculated; those of Experiment 2 were kept 7 days on diet I and 24 days on the experimental diets before being inoculated.

Biotin deficiency evidently had no effect on the total leucocyte count either before or after infection with the malaria parasites. In two other experiments there was likewise no indication of any effect of biotin deficiency on the differential leucocyte count (Table II).

The Changes in Biotin and FSF Content of the Plasma

Numerous experiments have confirmed the previously discovered fact (1) that the biotin content of the plasma of chickens and ducks increases during malarial infections. This increase was found to be an increase in the free biotin, that is, in the biotin activity measurable without preliminary hydrolysis of the plasma. The discovery of FSF presented the possibility that much or all of this increase in free biotin activity might really be an increase in free FSF liberated from the bound FSF present in normal plasma. That such is not the case has been shown by several experiments which involved measurement of

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the biotin activity of both unhydrolyzed and hydrolyzed plasma before and after extraction with ether. Complete data for one such series are given in Table III, and the results of this and 2 other series are summarized in Table IV. It is evident that the bulk of the higher free biotin activity of the plasma of infected animals is not ether-soluble and probably represents biotin itself. In no case has any significant amount of bound biotin been found. On the other hand, the free ether-soluble material is very small as compared to the bound material (bound FSF). The results in columns (*h*) and (*i*) of Table III illustrate the high degree of accuracy of the assays.

TABLE II

Differential White Blood Cell Counts in Biotin-Deficient (Diet IIa) and Control (Diet IIb) Chickens Just before and 5 Days after Inoculation with Plasmodium lophurae

(The chickens were 3 weeks old when inoculated.)

Experiment No.	No. chicks per group	Type of cell	Average*			
			Before inoculation		5 days after	
			Diet IIa	Diet IIb	Diet IIa	Diet IIb
			per cent	*per cent*	*per cent*	*per cent*
1	4	Lymphocyte	77.5	80.0	70.0	82.0
		Monocyte	6.5	4.0	8.0	7.0
		Heterophile	16.0	16.0	22.0	11.0
2	8	Lymphocyte	74.7	66.6	39.3	41.9
		Monocyte	6.8	8.6	18.4	18.8
		Heterophile	17.7	22.4	41.4	38.8
		Basophile	0.1	0.4	0.3	0.1
		Eosinophile	0.7	2.0	0.6	0.4

* For comparison, one may cite the following percentages based on 20 to 30 White Leghorn chicks 21 days old (15): lymphocytes 73.0, monocytes 3.5, heterophiles 18.0, basophiles 3.5, and eosinophiles 2.0.

When changes in biotin and FSF were being followed at frequent intervals in a group of animals it was not possible to make the 4 full determinations on each plasma sample. The 2 measurements which were made gave the total free activity, corresponding to column (*a*) of Table III, most of which is free biotin, and the bound activity, corresponding to column (*g*) of Table III, which represents exactly the bound FSF in terms of its biotin activity. In the plasma of uninfected control animals these activities fluctuated slightly in an irregular manner and showed no consistent sustained rise or fall. Repeated small bleedings, which had no effect on the hematocrit reading, and one large bleeding which was followed by a reduced hematocrit reading 2 days later, had no effect on the free biotin and bound FSF content of the plasma of uninfected animals. In some of the experiments with infected animals measurements were made

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TABLE III

Complete Assay Data for the Plasmas of 4 Ducks on the 3rd Day of a Severe Infection with P. cathemerium

Duck No.	Biotin activity as mγ per ml. plasma								
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*i*)
1	14.7	12.3	2.4	26.8	11.5	15.3	12.1	−0.8	12.9
2	13.0	10.3	2.7	27.2	11.8	15.4	14.2	1.5	12.7
3	11.0	9.5	1.5	26.5	8.8	17.7	15.5	−0.7	16.2
4	5.5	4.8	0.7	18.2	4.3	13.9	12.7	−0.5	13.2

(*a*) Values from plasma samples diluted in water.

(*b*) From samples diluted in water and shaken with ether. This represents the free biotin.

(*c*) (*a*) minus (*b*). This represents the free ether-soluble biotin-active material.

(*d*) From samples autoclaved in 3 N sulfuric acid at 15 pounds for 1 hour.

(*e*) From samples autoclaved in 3 N sulfuric acid and shaken with ether after neutralization. This would represent the total biotin.

(*f*) (*d*) minus (*e*). This represents the total ether-soluble biotin-active material.

(*g*) (*d*) minus (*a*). This represents the total bound material which is liberated by acid autoclaving.

(*h*) (*e*) minus (*b*). This would represent the bound biotin. The figures are essentially zero (within 10 per cent of the total bound material). There is no bound biotin.

(*i*) (*f*) minus (*c*). This represents the bound ether-soluble material (=FSF). The values agree within 10 per cent with the total bound material (column *g*).

TABLE IV

The Concentration of Biotin and of Ether-Soluble Factor (FSF) in the Plasma of Normal 40 Day Old Ducks and of Ducks of the Same Age at the Peak of a Malarial Infection

Condition of ducks	No.	Biotin activity as mγ per ml. plasma		
		Free biotin*	Free FSF‡	Bound FSF§
Not infected	1	1.8	1.0	11.3
	2	1.3	0.7	9.8
	3	1.8	0.2	9.3
	4	1.8	1.0	10.5
5th day of infection with *P. lophurae*	5	10.5	1.5	11.7
	6	3.3	0.2	8.5
	7	8.3	1.2	8.8
	8	5.0	0.5	7.8
3rd day of infection with *P. cathemerium*	9	12.3	2.4	12.9
	10	10.3	2.7	12.7
	11	9.5	1.5	16.2
	12	4.8	0.7	13.2

* Activity of unhydrolyzed plasma after ether extraction.

‡ Difference between activity of unhydrolyzed plasma before and after ether extraction.

§ Difference between activity of hydrolyzed plasma before and after ether extraction minus the free FSF.

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only once or twice during the course of the infection. Most of the results so obtained have fitted into the picture of events derived from the more complete series. The results of 2 such complete series, each involving 10 ducks, will now be considered in detail.

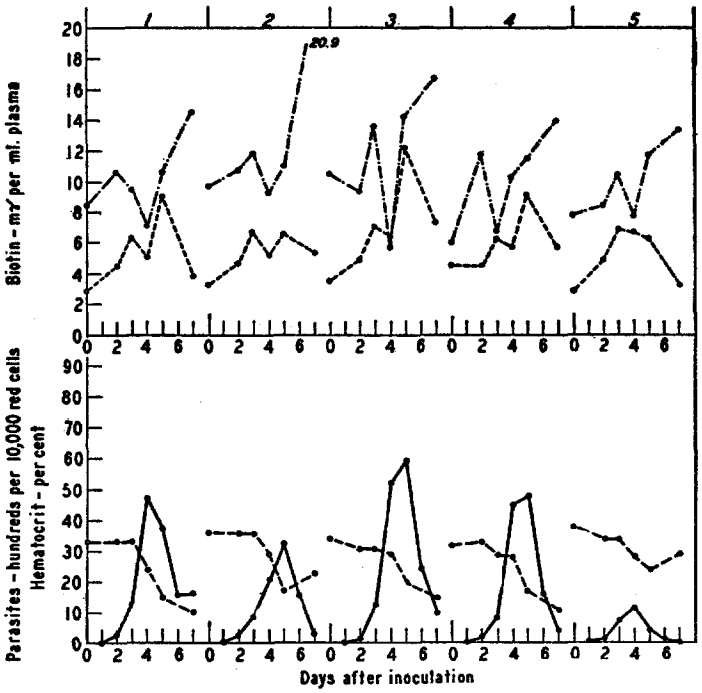

FIG. 2. The changes in parasite count (solid line) and hematocrit readings (long dash line), and in the plasma concentrations of free biotin (short dash line) and bound FSF (dot dash line), expressed in terms of its biotin activity after acid hydrolysis, in 5 ducks maintained on an adequate diet (diet I) and inoculated when 22 days old with *P. cathemerium*, 28 million parasites per 100 gm. of duck. From left to right—ducks 1 to 5.

In each of the 2 series, 5 ducks received the adequate diet I only, while the other 5 received in addition thrice weekly by mouth 15 γ of pure biotin. This very large biotin supplement had but little effect on the plasma level of free biotin and no effect on the level of bound FSF or on the course of the infections. The 10 ducks of the first series (here numbered 1 to 10) were inoculated with *P. cathemerium*. Plasma samples for biotin and FSF assay were taken just before and 2, 3, 4, 5, and 7 days after inoculation. The 10 ducks of the second series (11 to 20) were inoculated with *P. lophurae*. Plasma samples were taken just before and 4, 6, 7, 9, and 11 days after inoculation. The average weights of the ducks at the time of inoculation were, in grams: Nos. 1 to 5—207; Nos. 6 to 10—201; Nos. 11 to 15—206; Nos. 16 to 20—214.

The pertinent results for the individual ducks are shown in Figs. 2 to 5, and the averaged data are given in Tables V and VI. All of ducks 1 to 5 survived

their infection with *P. cathemerium* and all showed the same type of change in biotin and FSF concentration. The free biotin rose before any high parasitemia had been attained and before there was any anemia. It then dipped slightly, rose again, and finally fell toward normal values at the time when parasites were being rapidly removed from the blood and when the anemia was pro-

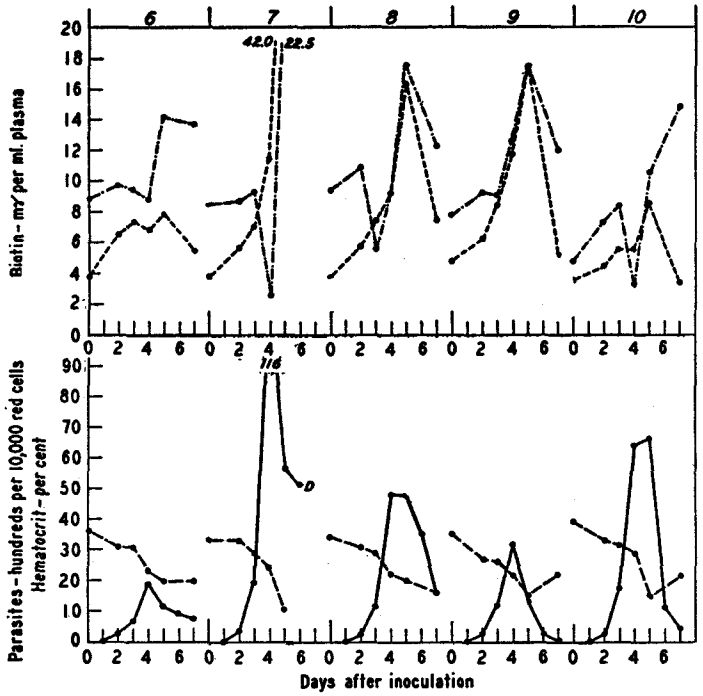

FIG. 3. The changes in parasite count (solid line) and hematocrit readings (long dash line), and in the plasma concentrations of free biotin (short dash line) and bound FSF (dot dash line), expressed in terms of its biotin activity after acid hydrolysis, in 5 ducks maintained on diet I plus 45 γ of additional biotin weekly administered by mouth. These ducks (ducks 6 to 10) were inoculated when 22 days old with *P. cathemerium*, 28 million parasites per 100 gm. of duck.

nounced. The bound FSF at first followed a similar course, except that its drop after the initial rise was greater. However, its second rise was sustained and the highest levels were reached at about the termination of the infection. Other experiments have shown that the bound FSF returns to normal within a week after recovery from the infection. The changes in ducks 6 and 8 to 10 were similar, except that the overall level of free biotin was somewhat raised. In duck 7, which died from the infection, both the free biotin and bound FSF were exceptionally high just before death. It is noteworthy that the normal relationship of excess bound FSF over free biotin was here reversed. This

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reversal was more strikingly illustrated in 4 of the 6 ducks which died of *P. lophurae* infection (Nos. 12, 15, 17, 18) in which the bound FSF, instead of continuing to increase fell sharply to zero or almost zero, while the free biotin rose to exceptionally high values. In the 4 ducks which survived their *lophurae* infections the changes in biotin and FSF were similar to those observed in the 9 ducks which survived their infections with *cathemerium*. The changes cer-

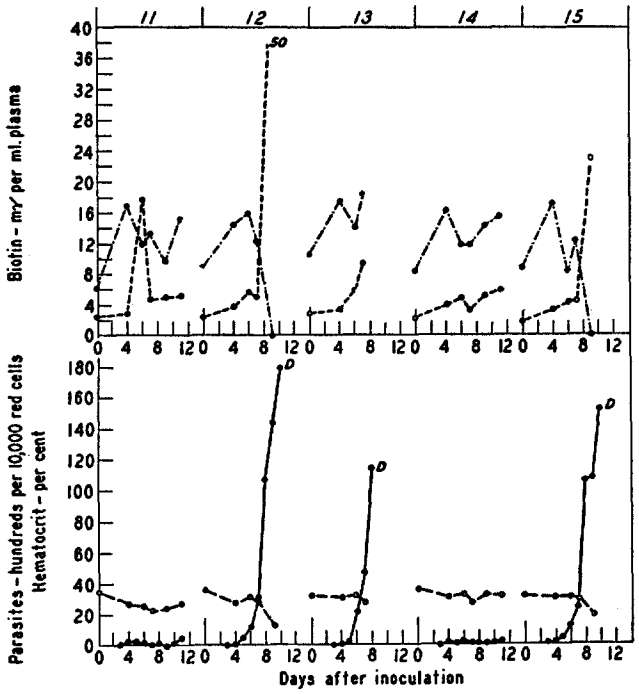

FIG. 4. The changes in parasite count (solid line) and hematocrit readings (long dash line), and in the plasma concentrations of free biotin (short dash line) and bound FSF (dot dash line), expressed in terms of its biotin activity after acid hydrolysis, in 5 ducks maintained on an adequate diet (diet I) and inoculated when 21 days old with *P. lophurae*, 4 million parasites per 100 gm. of duck. From left to right—ducks 11 to 15.

tainly show that the increase in free biotin is not associated with the development of an ability to control the multiplication of the parasites. The presence of a normal or above normal level of bound FSF seems to be associated with the development of resistance, while a low level of the material is associated with complete failure to cope with the parasites. The results with the 9 ducks which survived infection with *P. cathemerium* also indicate that a relatively low parasitemia was accompanied by a relatively great excess of bound FSF over free biotin, and conversely (compare ducks 1, 2, 4, and 5 with duck 3; duck 6

with ducks 8 and 10). Although data of this type are best considered for each individual animal, the general trend of the changes is also well shown by the averages given in Tables V and VI.

An experiment involving observations on immune ducks has furnished interesting data and at the same time provided a test of the reality of the changes observed in non-immune animals.

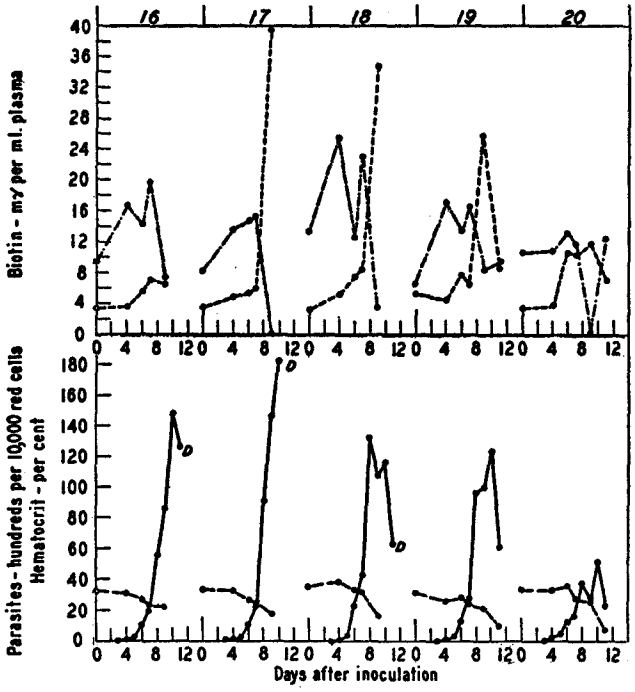

FIG. 5. The changes in parasite count (solid line) and hematocrit readings (long dash line), and in the plasma concentrations of free biotin (short dash line) and bound FSF (dot dash line), expressed in terms of its biotin activity after acid hydrolysis, in 5 ducks maintained on diet I plus 45 γ of additional biotin weekly administered by mouth. These ducks (ducks 16 to 20) were inoculated when 21 days old with *P. lophurae*, 4 million parasites per 100 gm. duck.

Five ducks (21 to 25) were used. Two of these had been inoculated when they were 13 days old with a small dose of *P. cathemerium* and had recovered from a light infection with this parasite. These and the 3 non-immune ducks, which had otherwise been treated in the same way, were all given a large dose of *P. cathemerium* when they were 38 days old. Plasma samples were taken just before and 1, 3, and 4 days after inoculation. The results are shown in Fig. 6.

The 3 non-immune animals underwent rapidly fatal infections, one being dead on the 4th and the other 2 on the 5th day after inoculation. There was hardly time for detailed increases and decreases in concentration of biotin and

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FSF to be observed, but the general change was clearly of the type previously observed in fatal infections with both *cathemerium* and *lophurae*—a sharp rise in free biotin before death, an initial rise followed by a drop in the bound FSF, with a reversal of the normal excess of FSF over biotin. The concentration of free biotin in the 2 immune ducks underwent no significant change. In both ducks the concentration of FSF showed a rise on the 1st day followed by a return to the original level in one and below the original level in the other.

TABLE V

The Average Concentrations of Free Biotin and Bound FSF (in Terms of Biotin Activity) in the Plasma of Ducks during the Course of Infection with P. cathemerium

Ducks	Diet		Time after inoculation, *days*					
			0	2	3	4	5	7
1–5	I (starting mash)	Free biotin, mγ per ml.	3.4	4.7	6.6	5.8	8.6	5.1
		Bound FSF, mγ per ml.	8.5	10.2	10.4	8.0	11.8	15.9
		Parasites per 10,000 red cells	—	200	990	3520	3630	670
6–10	I (starting mash) + 15 γ biotin 3 times per week	Free biotin, mγ per ml.	4.0	5.7	7.1	8.9	12.5*	5.3*
		Bound FSF, mγ per ml.	7.9	9.1	8.3	7.3	14.9*	13.2*
		Parasites per 10,000 red cells	—	280	1340	5560	2770*	720*

* Excluding data on one duck which died on 6th day.

Many more data would be needed to establish any connection between these changes in FSF and the immune response.

The Depletion of Liver Biotin Following Malarial Infection, and Preliminary Observations on the FSF Content of Liver, Spleen, and Lymph Glands.—It seemed reasonable to suppose that the increased free biotin present in the plasma of chickens and ducks infected with malaria was derived from various body tissues. Only the liver has been studied in this respect. As shown in Table VII, the biotin content of the livers of animals which had been fed a diet adequate in biotin and which had just undergone a malarial infection was only about half as great as that of control uninfected animals. Thus acute malarial infection does result in a notable withdrawal of biotin at least from the liver, a result in keeping with the hypothesis (16) that the recession of malignant growth observed during acute infections is a result of induced biotin deficiency.

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In biotin-deficient animals the biotin content of the liver was very low, but was actually somewhat higher in the infected than in the uninfected individuals. Perhaps in such animals the withdrawal of biotin from other tissues into the blood permits the deficient liver to seize and hold a portion of it.

TABLE VI

The Average Concentrations of Free Biotin and Bound FSF (in Terms of Biotin Activity) in the Plasma of Ducks during the Course of Infection with P. lophurae

Ducks	Diet		Time after inoculation, *days*					
			0	4	6	7	9	11
11–15	I (starting mash)	Free biotin, mγ per ml.	2.5	3.6	7.8	5.5*	20.7‡	5.6§
		Bound FSF, mγ per ml.	8.6	16.5	12.6	13.7*	6.0‡	15.5§
		Parasites per 10,000 red cells	—	160	1020	2110*	6310‡	320§
		Hematocrit, per cent	34	30	31	27*	22‡	30§
16–20	I (starting mash) + 15γ biotin 3 times per week	Free biotin, mγ per ml.	3.8	4.4	7.4	7.6	23.5‖	7.8§
		Bound FSF, mγ per ml.	9.6	16.7	13.6	17.2	3.8‖	10.8§
		Parasites per 10,000 red cells	—	160	1420	2630	9390‖	4160§
		Hematocrit, per cent	33	33	31	27	21‖	9§

* Including one duck which died on 8th day.
‡ Including two ducks which died on 10th day.
§ Data from two surviving ducks.
‖ Including three ducks which died on 10th day.

The preliminary results thus far obtained with measurements of FSF as well as biotin in various organs are summarized in Table VIII. It is apparent that while liver has a low content of FSF in proportion to its very high biotin content, spleen and lymph nodes have a relatively high content of FSF. This is of considerable interest in view of the relationship of these organs to the elaboration of plasma constituents and to mechanisms of resistance to infection.

The Effects of Biotin and Plasma Protein Fractions Rich in FSF on the Multiplication of P. lophurae in Vitro.—*P. lophurae* will multiply (17) in suspensions of duck red blood cells maintained *in vitro* in either the high potassium-red cell extract medium of Trager (18) or in the medium developed by Ball, Anfinsen,

Geiman, McKee, and Ormsbee (19) for the cultivation of *P. knowlesi* in monkey erythrocytes. The multiplication occurs exclusively within the red blood cells, so that the cultures are comparable to tissue cultures of viruses rather than to cultures of bacteria in non-living media. Since the medium of Ball *et al.* (19, 20) was simpler to prepare than the red cell extract medium, it was used for all of the following experiments.

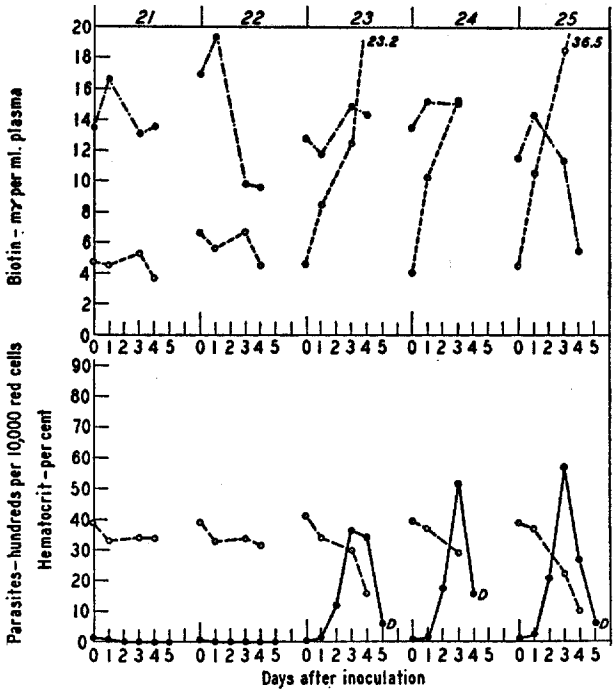

FIG. 6. The changes in parasite count (solid line) and hematocrit readings (long dash line), and in the plasma concentrations of free biotin (short dash line) and bound FSF (dot dash line), expressed in terms of its biotin activity after acid hydrolysis, in 5 ducks maintained on an adequate diet and inoculated when 38 days old with *P. cathemerium*, 200 million parasites per 100 gm. of duck. Left to right—ducks 21 to 25. Ducks 21 and 22 had recovered from a previous light infection with *P. cathemerium* and were immune.

The medium was modified by omitting biotin from the basal medium and by increasing the concentration of purines and pyrimidines, which were present in the final medium in the following amounts: cytidine and thymine each 1 mg. per l.; adenine, guanine, xanthine, and uracil each 2.5 mg. per l. Fifty ml. Erlenmeyer flasks equipped with gas inlet and outlet tubes were used as the culture vessels. Each flask received 4.5 ml. of culture medium plus 1.5 ml. of a mixture of a small amount of parasitized blood with a large amount of normal heparinized duck blood which had been concentrated by entrifuging it and drawing off about half of the plasma. This procedure gave in the final mixture about 1,000,000 red cells per c. mm. and about 500 parasites per 10,000 red cells. The flasks were held at 39–40°C. on a rocking ma-

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chine moving at 20 complete cycles per minute. A stream of moist 95 per cent air—5 per cent carbon dioxide was passed through them. Under these conditions the number of parasites increased 2 to 3 times in a 2 day period of incubation.

TABLE VII

The Effect of Biotin Deficiency and Malarial Infection on the Biotin Content of the Liver

Experiment No.	Host animal	Diet	Infection	No. animals	Average biotin mγ per gm. liver
1	Duck	IIa egg white	None	2	238
			P. cathemerium	2	307
		IIb casein	None	2	1663
			P. cathemerium	2	875
2	Chick	III synthetic	None	2	184
			P. lophurae	2	239
		III + biotin	None	4	1706
		I stock	None	4	1576
			P. lophurae	4	688
3	Chick	III synthetic	None	3	377
			P. lophurae	5	485
		III + biotin	None	3	2290
			P. lophurae	5	1916
		I stock	None	3	2530
			P. lophurae	4	990
4	Duck	IIb casein	None	4	1237
			P. cathemerium	4	840
		I stock	None	4	1513
			P. lophurae	4	1369

A wide variation in the concentration of free biotin in the culture fluid had no effect on the rate of multiplication (Table IX), a result in keeping with the presence of high concentrations of biotin in the plasma of ducks dying with heavy infections of *P. lophurae*.

Quite different results were obtained when certain human plasma protein fractions (secured through the kindness of Dr. L. E. Strong, Department of Physical Chemistry, Harvard Medical School, and prepared under a contract between Harvard University and the Office of Scientific Research and Development) were added to the culture medium. It has been previously reported (5)

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that fibrinogen, γ-globulin, and albumin from human plasma contain very little FSF while fractions containing the α- and β-globulins are relatively rich in it.

TABLE VIII

The Total Biotin and FSF Contents of Liver, Spleen, and Lymph Nodes

Animal	Organ	Biotin	FSF as mγ biotin activity per gm.
		mγ/gm.	
Chicken on adequate diet	Liver	1270	0
Chicken on adequate diet and infected with *P. lophurae*	Liver	676	0
Same as above	Liver	606	38
Same as above	Liver	636	0
Chicken on high egg white diet (II*a*)	Liver	106	36
Chicken on high egg white diet (II*a*) and infected with *P. lophurae*	Liver	162	52
Normal 2 month old duck on diet I	Spleen	49	52
	Lymph gland	53	53
	Lymph gland	44	35
Normal 2 month old duck on diet I	Spleen	73	79
	Lymph gland	77	67
	Lymph gland	71	108

TABLE IX

The Lack of Effect of High Biotin on the Development in Vitro of P. lophurae

Flask No.	Concentrations of free biotin* in culture fluid	Multiplication after 2 days‡
	mγ/ml.	
1	0.6	3.6
2	0.7	3.1
3	12.0	3.0
4	12.0	3.2
5	120.0	3.4
6	120.0	2.4

* Determined by assay of a sample of the final mixture of 1 part parasitized blood to 3 parts culture medium.

‡ The ratio of the number of parasites per 10,000 red cells after 2 days' incubation to the corresponding number before incubation.

When the latter fractions were added to the culture medium for *P. lophurae* at a concentration of 2 per cent they brought about hemolysis of most of the erythrocytes and concomitant degeneration of the malaria parasites. At lower concentrations they did not produce any evident hemolysis in the cultures, but they did inhibit the multiplication of the malaria parasites, while plasma

fractions containing little FSF did not (Table X). Cultures containing plasma fractions rich in FSF showed more parasites of a degenerate appearance than did control cultures. Similar antimalarial effects *in vitro* have been observed with bovine plasma fractions IV-1 and IV-3, 4 and with porcine plasma fractions III-O and IV-1 (obtained through the courtesy of Dr. J. B. Lesh of the Armour laboratories).

TABLE X

The Effect of Human Plasma Protein Fractions Relatively Rich in FSF on the Development in Vitro of P. lophurae

Experiment	Flask No.	Addition to basal culture medium	FSF content of added fraction as mγ biotin/gm. protein	Extent of multiplication after 2 days*
A	1	None	—	2.1
	2			1.9
	3	Fraction V-albumin 0.75 per cent	57	1.8
	4			2.3
	5	Fraction II-3 γ-globulin 0.75 per cent	57	2.5
	6			2.3
	7	Fraction IV-1,2 α-globulin 0.75 per cent	197	1.1
	8			1.2
B	1	None	—	3.0
	2			1.3
	3	Fraction V-albumin 1.1 per cent	57	2.1
	4			1.7
	5	Fraction II-1,2 γ-globulin 1.1 per cent	4	2.0
	6			1.5
	7	Fraction IV-1,1W α-globulin 1.1 per cent	280	0.9
	8			1.0

* The ratio of the number of parasites per 10,000 red cells after 2 days' incubation to the corresponding number before incubation.

DISCUSSION

I

In natural and especially in acquired immunity to avian malaria, phagocytosis of the parasites by the macrophages of the spleen and liver undoubtedly plays a major rôle (21). The decreased resistance to malaria of chickens ex-

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posed to x-rays (22) has been attributed to a destruction of lymphocytes, which are a source of new macrophages (21) and which are concerned in antibody formation (23). However, in the present studies, no indications were obtained that biotin deficiency in ducks and chickens affected either erythrocyte or lymphocyte formation. Biotin deficiency similarly has been found not to interfere with blood regeneration in rats (24).

The fact that the concentration of free biotin in the plasma increases during the malarial infections of chickens and ducks and then returns to normal if the animal recovers at first suggested that a relatively high concentration of biotin might in itself exert some antimalarial effect. This can hardly be so, since exceptionally high concentrations of biotin occur just before death in the plasma of animals which exhibit little resistance, and since a considerable range of biotin concentrations was not observed to have any effect on the extent of multiplication of *P. lophurae in vitro*. The presence of high concentrations of bound FSF at the termination of the patent infection in animals which recover, and of little bound FSF just before death in animals which do not recover, strongly indicates that the substance which yields FSF on hydrolysis is important in the resistance mechanism. Such an assumption is strengthened by the fact that plasma fractions relatively rich in FSF inhibit the growth *in vitro* of *P. lophurae*, while fractions poor in FSF do not. The fractions rich in FSF exert at higher concentrations a hemolytic effect, and it is interesting to note that in preliminary purification procedures the biotin growth activity of FSF and a hemolytic activity have gone together (5). In the acute malarial infections of birds and monkeys there occurs at the crisis an extensive destruction of uninfected as well as infected red cells, many of them being taken up by the macrophages of the spleen and liver (25). It is reasonable to suppose that this phagocytosis represents the ingestion of already injured cells (26), just as in trypanosome anemia it is probable that erythrocytes about to hemolyze are taken up by phagocytes (27). These facts fit the hypothesis that a lipoprotein, which on hydrolysis yields FSF, is present in plasma and, at suitable concentrations, can act both directly against malaria parasites and also in such a way as to aid their removal from the circulation by contributing to some sort of sensitization of the erythrocytes. This substance may be in part responsible for the generalized harmful serological effect on the parasites which occurs at the beginning of decline in parasite number and which has been described in detail for *P. brasilianum* in monkeys (28). An adequate tissue level of biotin must be assumed to be necessary for the formation and function of the lipoprotein, a not unreasonable assumption in view of the biotin-like growth activity of FSF. Verification of this hypothesis must await the chemical isolation of the active material in plasma and of the fat-soluble substance obtained from it by hydrolysis.

One observation has been made which may indicate a similarity between the biotin and FSF changes in avian malaria and in acute malaria of man.

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In a very severe case of *P. falciparum* malaria observed in New Guinea, in which 20 per cent of the red cells were infected and which would have been fatal but for the institution of vigorous parenteral drug therapy, the bound FSF content of the plasma was only 1.5 m γ of biotin activity per ml. and the free biotin content 6.0, as compared to a bound FSF content of 7.2 and a biotin content of 3.8 in the plasma of an uninfected individual assayed at the same time (Trager, unpublished). This is the same type of situation that we have observed in heavily infected ducks shortly before death—the free biotin is increased and the bound FSF considerably decreased.

The C'3 component of complement, which plays no part in complement fixation but which is necessary for immune hemolysis to occur (29), is a lipoprotein (30). Its possible relation to the material in plasma containing FSF activity has not yet been investigated. It was noted that the complement titer of the plasma of biotin-deficient chickens was as great as that of control chickens, and that the complement titer was decreased in chickens and ducks undergoing heavy infections with malaria. No quantitative relation could be established between complement titer and FSF content. This does not rule out a possible relation between C'3 and the material with FSF activity, since the complement titer may have been determined by the concentration of a component other than C'3 (29).

II

Biotin is no longer the only nutritional factor which has been found to influence the course of the parasitemia in experimental malaria. Riboflavin deficiency (12) and pantothenic acid deficiency (13) in chickens inhibit the multiplication of the parasites, as does vitamin C deficiency in monkeys (31); while a deficiency of folic acid (11), protein (32), or nicotinic acid (33) in chickens, like biotin deficiency, decreases the resistance of the host. Since folic acid deficiency is accompanied by anemia and leucopenia (15), the higher relative parasite counts observed may have been partly a result of the smaller number of red cells and partly a result of a depletion in the phagocytic cells. Protein deficiency may exert its effect by way of an interference with antibody formation (34). However, protein deficiency so severe as to limit the weight of the chickens to 50 gm. at 5 weeks of age may also have interfered with a variety of resistance mechanisms all of which probably involve proteins of one sort or another. It has recently been shown that rats fed very low protein diets, similar to the diets used in the experiments with protein deficiency and susceptibility to *P. lophurae*, had such low levels of biotin and certain other B vitamins in the liver as to indicate frank or incipient deficiencies (35).

Brooke (36) has observed dietary effects on the course of *P. relictum* and *P. cathemerium* in canaries and *P. relictum* in pigeons. A diet of restricted quantity decreased resistance. It is likely that multiple deficiencies were concerned.

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SUMMARY

Biotin deficiency produced by a synthetic biotin-deficient diet was as effective in decreasing the resistance of chickens to infection with *Plasmodium lophurae* as biotin deficiency produced by a diet high in egg white.

In moderately biotin-deficient ducks *Plasmodium cathemerium* at first multiplied more slowly than in adequately fed controls. The parasitemia in the deficient animals later overtook that in the controls and attained higher peak parasite numbers. The multiplication of *P. cathemerium* was notably inhibited in ducks inoculated when they were approaching death from biotin deficiency.

The total and differential leucocyte count of biotin-deficient chickens did not differ significantly from that of adequately fed controls.

During infection of chickens with *P. lophurae* and ducks with either *P. lophurae* or *P. cathemerium* significant changes occur in the concentration in the plasma of free biotin and of a material which on hydrolysis yields a fat-soluble substance (FSF) having the biological activities of biotin but differing chemically from it. In ducks which survived infections with *P. cathemerium* or *P. lophurae* the biotin concentration rose very early in the course of the infection, before there was any anemia. It fell slightly, rose to a peak at about the time of the peak parasite number, and then returned to normal. The concentration of bound FSF, which was determined in terms of its biotin activity, increased at first, then decreased, then rose and continued at a high level throughout the period of decline in parasitemia. In most of the animals which died of either infection the free biotin, instead of returning to normal, rose to very high values just before death, while the bound FSF, instead of remaining at a high value, fell to very low values, reaching zero in several animals. Greater resistance seemed to be associated with a greater excess of bound FSF over free biotin. In animals about to die the free biotin exceeded the bound FSF.

The biotin content of the liver of ducks and chickens fed an adequate diet and killed just after having undergone an infection with either *P. lophurae* or *P. cathemerium* was much less than that of control uninfected animals.

When *P. lophurae* was cultured *in vitro* in suspensions of duck erythrocytes a very wide range in biotin concentration in the culture fluid did not affect its rate of multiplication. Plasma protein fractions relatively rich in FSF at a concentration of 0.75 per cent inhibited multiplication, while comparable concentrations of plasma fractions poor in FSF did not.

The results obtained fit the assumption that the substance in plasma which yields FSF is directly concerned in resistance to avian malaria.

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